

Effects of temperature, soil ammonium concentration and fertilizer on activity and community structure of ammonia oxidizers

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Dedicated to my family

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Abbreviations

AMO	Ammonia monooxygenase
<i>amoA</i>	Alpha subunit of the ammonia monooxygenase
Anammox	Anaerobic ammonium oxidation
APS	Ammoniumperoxodisulfate
ATCC	American Type Culture Collection
bp	Basepairs
DGGE	Denaturant Gradient Gel Electrophoresis
DNRA	Dissimilate nitrate to ammonium
e ⁻	Electron
EDTA	Ethylendiaminetetraacetic acid
FISH	Fluorescence In Situ Hybridization
GC-clamp	Guanosine-cytosine clamp
GC	Gas chromatograph
gdw	Gram dry weight
GeneBank	Nucleotide sequence database - http://www.ncbi.nlm.nih.gov/Entrez/
HAO	Hydroxylamine oxidoreductase
IPTG	Isopropyl-β-D-thiogalactoside
LB	Luria Broth
MPN	Most probable number
O.D.	Optical Density
PCR	Polymerase Chain Reaction
PVPP	Polyvinylpolypyrrolidone
RFLP	Restriction Fragment Length Polymorphism
rpm	Rounds per minute
SDS	Sodium Dodecyl Sulfate
sp.	Species (single)
spp.	Species (plural)
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethyldiamine
T-RFLP	Terminal Restriction Fragment Length Polymorphism
Tris	Tris (hydroxymethyl)-aminomethane
WHC	Maximal water holding capacity
w/w	Weight per weight
wt/vol	Weight per volume
X-Gal	5-Bromo-4-Chloro-3-indolyl-β-D- galactopyranoside

I. Introduction

Nitrification, the conversion of the most reduced form of nitrogen (NH_3 ammonia), to its most oxidized form, (NO_3^- nitrate), plays an important role in the nitrogen cycle of various ecosystems including soils (Prosser, 1989). Nitrification has a great impact on environmental processes, such as acidification of soils (Prosser, 1989; Biederbeck et al., 1996) and biodeterioration of building materials (Meincke et al., 1989). Loss of nitrogen from fertilized agriculture soils could lead to leaching of nitrite and nitrate since, as negative ions, these are more mobile than ammonium, and therefore contamination of aquifers, springs and other drinking water sources is possible (Bauhus and Melwes, 1991). Nitrification is also known to produce nitric oxide (NO) and nitrous oxide (N_2O) as by-products, which are well known greenhouse gases and ozone scavengers (Crutzen, 1970; Dickinson and Cicerone, 1986). Concurrently, nitrification has important positive effects, since high concentrations of ammonium are toxic for life (Arthur et al., 1987) and create a large oxygen demand. Nitrification therefore prevents eutrophication of surface and ground water from high input of fertilizer (Hall and Jeffries, 1986), and also prevents the growth of phototrophs and heterotrophs, which could lead to a decrease of biodiversity and the creation of anoxic conditions. Nitrification can also be useful against anthropogenic damage to the environment, by reducing the ammonium content of wastewater in sewage treatment before discharge into aquatic environments (Painter, 1986).

Nitrification is composed of two stages. Ammonia oxidizers are involved in the first step, when ammonia is oxidized to nitrite, and nitrite oxidizers are involved in the second step, when nitrite is oxidized to nitrate (Prosser, 1989) (Fig. 1). Ammonia oxidation is thought to be the rate-limiting step for nitrification in most systems, as nitrite is rarely found to accumulate in the environment (Prosser, 1989; De Boer et al., 1990; 1992).

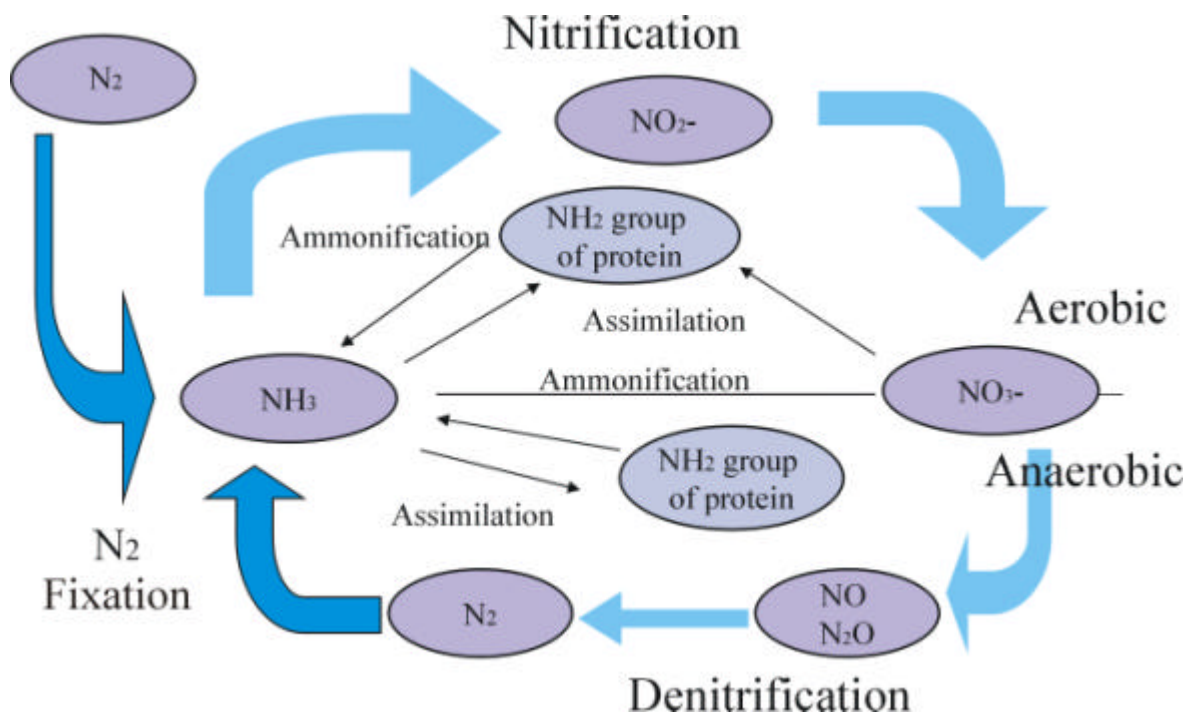


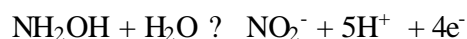
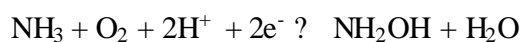
Figure 1: The nitrogen cycle

Nitrification is followed by denitrification, which is the production of di-nitrogen (N_2), nitric oxide (NO) and nitrous oxide (N_2O) under anaerobic conditions. Interactions between nitrifiers and denitrifiers are often mediated across oxic/anoxic interfaces such as soil aggregates, which involve the diffusion of substrates and products from oxic to anoxic niches (Zausig et al., 1993). Another possible process is chemodentrification, but it is not clear to what extent this process affects nitrogen losses in natural environments (Kowalchuk and Stephen, 2001). Release of di-nitrogen allows the biological fixation of nitrogen to ammonium (NH_4^+), which is the available form of nitrogen for all other microorganisms. Another process, where ammonium is released to the environment, is ammonification (or mineralization) of organic nitrogen compounds. The majority of ammonium uptake is due to assimilatory processes by most microorganisms in the environment. These are the main competitors for ammonia oxidizers, which are involved in dissimilatory process (Bock et al.,

1986). Furthermore, ammonia is highly volatile at a high pH and therefore readily lost from alkaline environments. In such cases, nitrification may facilitate nitrogen retention by oxidizing ammonia to less volatile nitrogen forms and thus makes them be available for assimilation (Kowalchuk and Stephen, 2001).

1. The autotrophic ammonia oxidizers

Oxidation of ammonia under aerobic conditions is mainly performed by the chemolithotrophic ammonia oxidizers, which are able to use the energy released during the oxidation to nitrite for growth and maintenance as well as for the fixation of carbon dioxide (CO₂) into cell material. The first stage of ammonia oxidation is the conversion to hydroxylamine, which is carried out by a membrane-bound ammonia monooxygenase (AMO), while the substrate is ammonia (NH₃) rather than ammonium (NH₄⁺) (Suzuki et al., 1974). This reaction alone does not generate ATP. The second reaction, in which hydroxylamine is oxidized to nitrite (via un-characterized intermediates), by the soluble enzyme hydroxylamine oxidoreductase (HAO) (Fig. 2), is coupled to electron transport and ATP generation:



Many chemolithoautotrophic ammonia oxidizers have been isolated from a wide range of soils, freshwater and marine environments and validly described (Watson et al., 1971; Watson and Mandel, 1971; Harms et al., 1976; Koops et al., 1976; Jones et al., 1988; Koops et al., 1990; 1991; Ustaker et al., 1996). These isolates are obligatory aerobic, gram-negative bacteria, while the known pure cultures optimally grow in pH values of 7.5-8.0 (upper and lower limits are at about 6.0 and 9.0, representatively), and with ammonium concentrations ranging from 2 to 50 mM at 25-30°C. The tolerance against increasing ammonia concentrations differs markedly between the various species. Despite the inability to grow on organic substrates, some of the species have the ability for hydrolysis of urea and use it as a

primary ammonia source. The doubling time of ammonia oxidizers ranges from 8 hours to several days, and therefore ammonia oxidizers are slow growing bacteria (Bock et al., 1986; Koops and Möller, 1992).

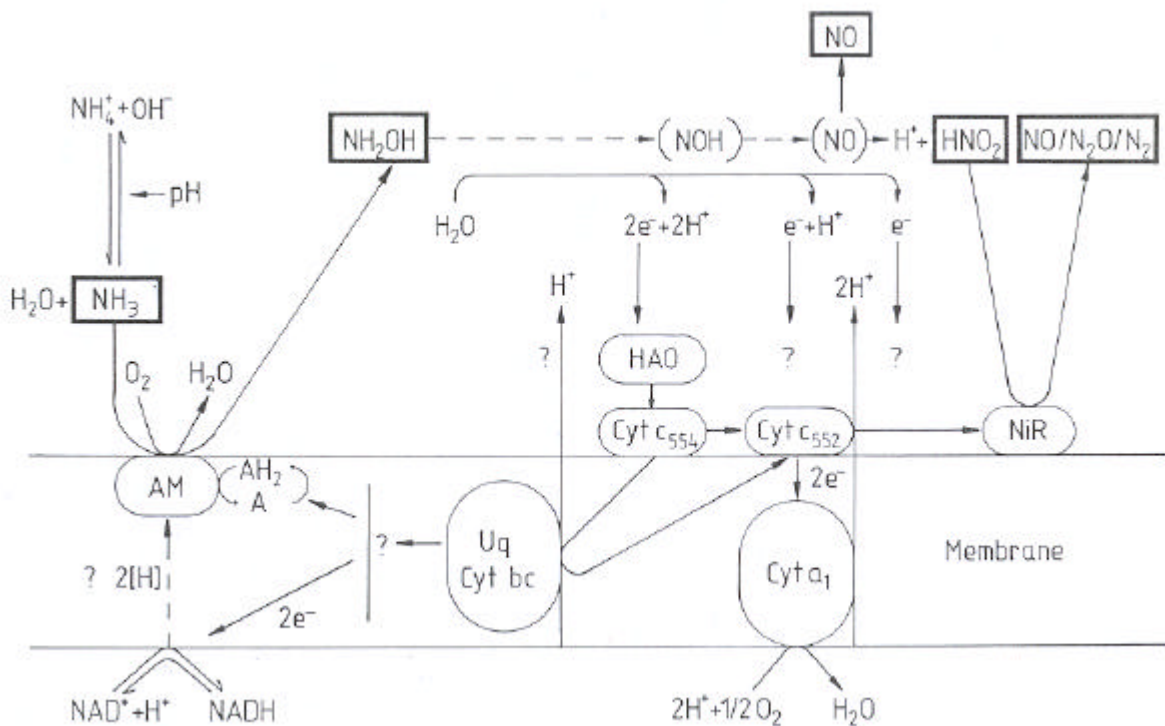


Figure 2: Hypothetical model of electron flow from hydroxylamine oxidation to nitrite and the coupling of ammonia hydroxylation, NADH generation, nitric oxide production, and nitrite reduction. AM ammonia monooxygenase, HAO, hydroxylamine oxidoreductase, NiR, nitrite reductase; Cyt, cytochrome; and Uq, ubiquinone (Bock et al., 1992).

In summary, most of the pure cultures grow well in a liquid batch at moderate temperatures (25-30°C), at a narrow pH range from neutral to slightly alkaline and at low to moderate salt concentrations (Koops and Möller, 1992). Oren, (1999) explained these restrictions by the relatively low growth efficiency of nitrification reactions, which might not allow these bacteria to survive in energetically expensive extreme environments. However, ammonia oxidizers have been found in various environments including extreme habitats such as alkaline soda biotopes (Sorokin, 1998), Antarctic ice (Arrigo et al., 1995), hot springs

(Golovatcheva, 1976) and in association with marine sponges (Diaz and Ward, 1997). Recently an isolate of ammonia oxidizers was retrieved from a Mongolian alkaline soda lake, which combined two extreme environmental factors, i.e. alkaline pH and high salinity (Sorokin et al., 2001). This isolate was active at high salinity (0.1 to 1.0 M of total Na⁺, optimum 0.3 M), and at pH values of up to 11.3 (optimum at 9.5 to 10), but showed > 99% similarity by its 16S rRNA sequence to *Nitrosomonas halophila*, and therefore was not described as a new species.

Another isolate, which showed adaptation to extreme acidic conditions after exposure to pH fluctuation, is *Nitrosospira* AP. AHB1 (De Boer et al., 1995). Furthermore, the use of inhibitors, such as acetylene and nitrapyrin, indicates that autotrophic ammonia oxidizers are the most important nitrifying bacteria in acidic soils (Stams et al., 1990; De Boer et al., 1991; 1992). These bacteria were found in a wide range of acidic soils including agricultural fields, tea plantations, coniferous and deciduous forests, heathlands and natural soils (Weber and Gainey, 1962; Walker and Wickramasinghe, 1979; Robertson, 1982a; 1982b; Troelstra et al., 1990; Pennington and Ellis, 1993), and even in soils at a pH value as low as 3.0 (Robertson, 1982b; De Boer et al., 1992). Although low soil pH does not seem to exclude nitrification in vegetated soils, there are also many soils in which nitrification was not detected (Robertson, 1982b). Nitrification in acidic soils could be explained to some extent by growth on surfaces (Allison and Prosser, 1993) or in aggregates (De Boer et al., 1991), by ureolytic activity (De Boer and Laanbroek, 1989; Allison and Prosser, 1991b) and by heterotrophic nitrification (Killman, 1986). An additional explanation is the existence of strains adapted to low-pH environments (De Boer et al., 1995; Stephen et al., 1996).

2. Temperature effect

Two other environmental factors that seem to have the most dramatic influence on ammonia oxidizer populations are temperature and moisture (Belser, 1979). Water content

has been reported to stimulate nitrification up to an optimum, where oxygen diffusion becomes limiting (Schrödt and Tietjen, 1971). Temperature influences the ammonification rates (mineralization), and a Q_0 of about 2.0 has been reported (Stanford et al., 1973). Therefore, at different temperatures release of ammonium will be different. Moreover, there is considerable evidence that high temperature would inhibit autotrophic nitrification more than ammonification (Belser, 1979). Keeney and Bremner, (1967), who tested several soils, observed that at 40°C nitrification was completely inhibited, while ammonification increased with increase of temperature at all tested temperatures (up to 40°C). However, there were also exceptions like in a tropical Australian soil, which showed optimal nitrification at 35°C and optimum ammonification rates at about 50°C (Myers, 1975). In this soil, nitrification activity was observed up to 60°C, well above the maximum temperature reported for temperate soils. Similar observation were shown by Mahendrappa et al. (1966), who found that indigenous ammonia oxidizers had temperature optima adapted to their climate region. However, at low temperatures, both ammonification and nitrification rates are greatly reduced (Belser, 1979). Furthermore, Malhi and McGill, (1982) compared soils from the tropics (Australia, 25°C mean annual temperature), temperate zone (Iowa, USA, 10°C mean annual temperature) and northern latitudes (Alberta, Canada, 2.5 °C mean annual temperature). They showed that the optimum temperatures for communities from these soils were 35°C, 30°C and 20°C, respectively. However, Stark and Firestone, (1996) found that temperature optimum of communities from different soil microsites appeared to be more related to differences in temperatures at particular times of the year than to differences in mean annual temperatures. Furthermore, optimum temperature for communities beneath oak canopies (31.8°C) was lower than in open interspaces (35.9°C). Interestingly, Quinlan, (1980) demonstrated that the optimum temperature for nitrification increased with the increasing of ammonium concentration.

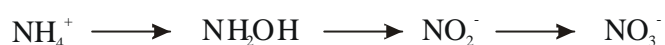
3. Ammonia oxidizers under anaerobic conditions

Until recently ammonia oxidation was considered as an obligatory aerobic process (Abeliovich, 1987). However, some ammonia-oxidizers cope with low oxygen concentrations (Bodelier et al., 1996; Kowalchuk et al., 1998; Bothe et al., 2000). Furthermore, *Nitrosomonas europaea* was found in anaerobic areas of wastewater reservoirs, and were able to use nitrite as electron acceptor if pyruvate was provided as an energy source (Abeliovich and Vonshak, 1992). *Nitrosomonas eutropha* was found to be able to grow under anaerobic conditions (Schmidt and Bock, 1997). *Nitrosomonas europaea* and *Nitrosomonas eutropha* could also simultaneously nitrify and denitrify during growth under oxygen limitation, by reducing nitrite or nitrogen dioxide using molecular hydrogen as electron donor (Poth, 1986; Bock et al., 1995). Although this process is not thought to support cell growth, it may provide sufficient energy to allow survival under anaerobic condition. Furthermore, Zart and Bock, (1998) showed that *Nitrosomonas eutropha* exhibited a higher aerobic denitrifying, nitrification rate, growth rates and maximum cell density with supplement of nitrogen dioxide (NO₂) or nitric oxide (NO), but not a higher growth yield.

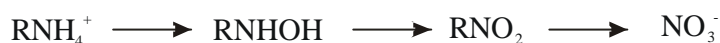
4. Other nitrifying bacteria

Recently, another process of an obligatory anaerobic ammonia oxidation was described and called Anammox. van de Graaf et al. (1995) found that ammonium was converted to dinitrogen via intermediates hydroxylamine and hydrazine, accompanied by the disappearance of nitrite ($\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O}$). The organisms that were found to be involved in this process are members of the order *Planctomycetes*, but until now there are no representative pure cultures of the involved bacteria (Strous et al., 1999; Schmidt et al., 2002). Although, these bacteria probably exist where ammonia and nitrite coexists in anaerobic conditions, their distribution in nature is still not understood. Recently, Thamdrup and Dalsgaard, (2002) reported that up to 67% of the N₂ formation in continental shelf sediments was due to anaerobic ammonia oxidation and only 33% was due to denitrification.

Another group, which oxidizes ammonia to nitrite or nitrate under aerobic conditions, includes various species of prokaryotes and eukaryotes, such as fungi, algae, cells from animal tissue (like rat liver) as well as heterotrophic bacteria (Tate, 1977; Focht and Verstraete, 1977; Lettl, 1985; Killman, 1986; Lang and Jagnow, 1986; Kuenen and Robertson, 1987). The heterotrophic nitrifiers do not gain energy by the oxidation reactions. There are at least two distinct pathways involved:



The inorganic pathway of nitrification



The organic pathway of nitrification

Moreover, heterotrophic nitrifiers use a wide range of substrates like aliphatic, aromatic, halogenated molecules (Hooper et al., 1997), nitrite, hydroxylamine and nitro-aromatic compounds (Kuenen and Robertson, 1994), which probably serve different functions such as synthesis of chelating hydroxamates under conditions of iron limitation (Verstrae and Alexander, 1973). Most of the heterotrophic nitrifiers are also denitrifiers (Castignetti and Hollocher, 1984; Robertson and Kuenen, 1988), and some of them are also able to denitrify aerobically (Kuenen and Robertson, 1987; Robertson and Kuenen, 1992). Although in few studies of acidic coniferous forest soils heterotrophic nitrification was found to be the dominant process (Schimel et al., 1984; Killman, 1986; 1990), in most cases the contribution of heterotrophic nitrifiers to total nitrification is thought to be small (Schmidt, 1982; Prosser, 1989). Since in the present study I used aerated soils including an agriculture soil and three meadow soils, I focused on the aerobic autotrophic ammonia oxidizers.

5. Phylogenetic affiliation

The existing cultures of aerobic ammonia oxidizers comprise two monophyletic groups based on their 16S rRNA gene sequences. One group belongs to the α -*Proteobacteria* with *Nitrosococcus oceani* and *Nitrosococcus halophilus* as the only known species. The other group belongs to the β -*Proteobacteria* and includes two genera: *Nitrosomonas* and *Nitrospira* (Woese et al., 1984; 1985; Head et al., 1993; Teske et al., 1994). The traditional identification, which is based on morphological characteristics, defines *Nitrosovibrio*, *Nitrospira* and *Nitrosolobus* as separate genera while molecular analysis has reclassified them all as the genus *Nitrospira* (Head et al., 1993). *Nitrosomonas* species have also been reclassified based on DNA homologies, GC content of the DNA, shape and ultrastructure of the cells, salt requirements, ammonia tolerance, utilization of urea as ammonium source and whole cell protein patterns (Koops et al., 1991). Ammonia-oxidizing bacteria and environmental sequences belonging to the β -*Proteobacteria* were divided into 7 to 9 clusters based on 16S rRNA gene sequences (Stephen et al., 1996; Purkhold et al., 2000). Clusters 0 to 4 belong to *Nitrospira* spp., and clusters 5 to 8 consist of *Nitrosomonas* spp.. *Nitrospira* cluster 1 and *Nitrosomonas* cluster 5 are characterized only by environmental sequences and have no representative pure cultures.

6. Culture methods for studying the ammonia oxidizer community in natural environments

Most studies on ammonia oxidizers were performed with *Nitrosomonas europaea*, which was easy to obtain from international bacterial culture collection (Watson et al., 1989). However, it was suspected that *Nitrosomonas europaea* does not represent the diversity that exists in natural habitats. Ammonia oxidizers have been recovered from a wide variety of environments (Prosser, 1986). Ammonia oxidizers are slow growing bacteria due to their low energy yield, and therefore their isolation is a time-consuming task. Furthermore, cell yield is poor, colony development on solid medium typically takes several months, colonies are small

and difficult to transfer, and elimination of heterotrophic contaminations is often difficult because of the higher growth rates of contamination (Allison and Prosser, 1992). Other cultivation-based methods, such as most probable number (MPN) counting (Matulewich et al., 1975) and selective plating (Ford et al., 1980) are difficult for the same reasons and cause poor efficiency (Belser, 1979), which leads to numbers that are not representative for the real diversity and abundance of ammonia oxidizers in the environment (Hiorns et al., 1995; Stephen et al., 1996). Many bacterial cells present in natural populations appear viable and yet cannot be detected by traditional isolation procedures (Kogure et al., 1979; Roszak and Colwell, 1987). The use of lipid profiles has failed to provide a satisfactory discrimination for the genera, which have been examined (Blumer et al., 1969). Ammonia oxidizers account for a very small proportion of the total bacteria population in natural environments. Sensitive and specific alternative methods for their identification were necessary in order to study their ecological importance in natural systems. Immunoassay fluorescent polyclonal antibody-based techniques have been used to study serological diversity and species distribution in soil (Belser and Schmidt, 1978), sewage (Smorczewski and Schmidt, 1991), lake sediments (Takahashi et al., 1982; Völsch et al., 1990) and marine environments (Ward, 1982; Ward and Carlucci, 1985; Ward, 1986; 1987). This method has advantages compared to MPN analysis, such as lower standard deviation, higher sensitivity for small populations and the possibility to follow population dynamics. However, it also requires initial isolation of pure cultures in order to raise antibodies for subsequent detection assays. Since ammonia oxidizers are slow growing bacteria and require months and even years for isolation and purification, development of detection methods that are culture-independent would be advantageous.

7. The molecular approach and 16S rRNA gene analysis

The molecular approach based on cultivation-independent methods was a promising alternative to study the diversity of ammonia oxidizers in the environment. The fact that ammonia oxidizers seem to have only one operon of the 16S rRNA gene (Aakra et al.,

1999a), simplifies the work with this gene as a molecular marker. Utaker and Nes, (1998) evaluated all the 16S rRNA oligonucleotides, which were available at the time of their study (Nejidat and Abeliovich, 1994; McCaig et al., 1994; Hiorns et al., 1995; Voytek and Ward, 1995; Pommerening-Röser et al., 1996; Hovanec and Delong, 1996; Mobarry et al., 1996; Kowalchuk et al., 1997; Hastings et al., 1997; Ward et al., 1997). The application of specific oligonucleotides for detection of bacterial strains in the environment has some limitations, which must be considered (Stahl and Amann, 1991; Amann et al., 1995; 1996). Most importantly, the majority of oligonucleotides are made on the basis of a relatively small number of DNA sequences, usually originated from pure cultures, which in most cases do not represent the true diversity of bacteria in the environment (Wagner et al., 1993). This in particular is true for the ammonia oxidizers, because of the low number of the pure cultures available for these bacteria. Consequently, there is always a risk that oligonucleotides fail to discriminate between specific and unspecific targets (Amann et al., 1995; 1996), especially when working with environmental samples containing complex microbial gene pools. However, improvement of the existing oligonucleotides is a dynamic process due to isolation of new species (Aakra et al., 1999b) and retrieval of new sequences from different primer sets of different environments. Other biases could arise from cell lysis efficiency, extra-cellular DNA or RNA, the fidelity of the Taq-polymerase and quality of the template molecule (Kopczynski et al., 1994; Amann et al., 1995). Oligonucleotides could be used as PCR primers for amplification of 16S rDNA or functional genes, which could only give qualitative information but no quantification of cells number or activity. PCR products then are cloned or analysed by fingerprinting method like restriction fragment length polymorphism (RFLP) (Aakra et al., 1999a), terminal restriction fragment length polymorphism (T-RFLP) (Horz et al., 2000), or denaturant gradient gel electrophoresis (DGGE) (Kowalchuk et al., 1997; Stephen et al., 1998; Oved et al., 2001). A good alternative method for quantification of whole cell numbers is Fluorescence In Situ Hybridization (FISH), which is especially good for studying the diversity and distribution of microorganisms in their natural environments

(Wagner et al., 1995; 1996; Juretschko et al., 1998). Abundances derived from FISH compared well with those estimated by antibody immunofluorescence; slight apparent differences in cell distribution were attributed to the slight different specificities of the antibodies versus the DNA probes (Voytek et al., 1999).

Another quantification method is 16S rRNA slot-blot hybridization, for which different probes are designed for ammonia oxidizers in general for genus-levels and the species-level (Hovanec and Delong, 1996; Mobarry et al., 1996). However, all hybridization methods are based on known sequences and therefore cannot detect novel species in the environment. In order to detect unknown species, a method where less specific conditions are applied, such as PCR, is more appropriate. Smith et al. (2001) studied the diversity of marine and soil environments both by cloning and sequencing from environmental samples and by enrichment cultures derived from the same environments. Although there was no evidence for selection, there were significant differences in species composition assessed by molecular and cultivation-based approaches in soils. While in enrichment cultures *Nitrosospira* cluster 3 sequences were more abundant, environmental sequences were distributed between *Nitrosospira* cluster 2, 3 and 4. However, in marine environments the majority of enrichment cultures contained *Nitrosomonas*, whereas environmental sequences belonged to *Nitrosospira* species. In spite of these differences, isolation of ammonia oxidizers identified by molecular methods is still very important to obtain a more comprehensive picture of their phenotypes.

8. The ammonia monooxygenase (AMO) as a molecular marker

The 16S rRNA sequence similarities among different ammonia oxidizers are so high that only limited phylogenetic information can be obtained using this gene as a molecular marker (Aakra et al., 1999a; Purkhold et al., 2000). A good alternative for 16S rRNA as a marker gene is comparative sequence analysis of *amoA*, which provides valuable additional

information (Rotthauwe et al., 1997). The *amoA* gene, a membrane-associated active site polypeptide, codes for the alpha subunit of the ammonia monooxygenase (AMO). AMO is the key enzyme of all aerobic ammonia oxidizers, catalysing the oxidation of ammonia to hydroxylamine. PCR primers were developed for amplification of *amoA* from environmental samples as a functional marker (Sinigalliano et al., 1995; Rotthauwe et al., 1997; Webster et al., 2002). The phylogeny of the *amoA* gene was found to largely correspond to the phylogeny of the 16S rRNA gene (Purkhold et al., 2000; Kowalchuk and Stephen, 2001; Aakra et al., 2001). The AMO has three subunits; AMO-C, AMO-A, and, AMO-B with different sizes (31.4 kDa, 27 kDa, and 38 kDa, respectively), structures and arrangements within the membrane/periplasmic space of the cell (Hooper et al., 1997). The three subunits of AMO from autotrophic ammonia oxidizers are encoded by the genes: *amoC*, *amoA*, and *amoB* of the *amo* operon (Sayavedra-Soto et al., 1998; Alzerreca et al., 1999). All three AMO subunits of several pure cultures of ammonia oxidizer genes were cloned and sequenced (McTavish et al., 1993a; 1993b; Bergmann and Hooper, 1994; Rotthauwe et al., 1995; Klotz et al., 1997). The *amoA* gene exists in multiple copies in the genome of ammonia oxidizers belonging to the β subdivision of the *Proteobacteria* (Norton et al., 1996). *Nitrosomonas* strains usually carry two gene copies whereas most *Nitrospira* strains carry three (Klotz and Norton, 1998), but only one copy was found in one of the two species that belongs to the γ subdivision of the *Proteobacteria*, *Nitrosococcus oceani* (Alzerreca et al., 1999). Most multiple gene copies are nearly identical. While the similarity between *amoA* from different species is relatively low, it is unlikely that the multiple gene copies originated from horizontal gene transfer. Instead, they probably arose by gene duplication events occurring relatively early in the evolution of this lineage (Norton et al., 1996; Klotz and Norton, 1998). Direct sequencing of *amoA* genes from 31 pure cultures amplified by the primer set of Rotthauwe et al. (1997) showed unambiguous sequences, which indicate that using this primer set will not overestimate the diversity in the environment (Aakra et al., 2001). The *amoA* primer set is highly specific for ammonia oxidizers and is suitable for assessing community shifts

(Rotthauwe et al., 1997; Horz et al., 2000; Oved et al., 2001; Nicolaisen and Ramsing, 2002; Avrahami et al., 2002). Nicolaisen and Ramsing, (2002) reported multiple DGGE bands due to the two wobble positions in the amoA reverse primer, i.e. amoA-2R (Rotthauwe et al., 1997). However, their environmental samples mainly included the detection of *Nitrosomonas* species. Oved et al. (2001) on the other hand, did not observe multiple bands in their samples, which contained both *Nitrosomonas* and *Nitrospira* species. Nevertheless, it seems to be desirable to check whether non-degenerate amoA primers would give better results.

9. Community structure of ammonia oxidizers in different natural environments

The first studies, which used molecular tools, revealed that *Nitrospira* spp. were ubiquitously present and also the dominant ammonia oxidizers in most natural environments. However, *Nitrosomonas* spp. could also be detected in many environment samples. Hiorns et al. (1995) studied various environments and suggested that *Nitrospira*-like organisms are more widespread in the environment than *Nitrosomonas* species. Furthermore, Hastings et al. (1998) studied the water column and sediment of a eutrophic lake during the seasonal stratification cycle and found only *Nitrospira* species, while *Nitrosomonas* species could be retrieved only by enrichment cultures, in agreement with the observation of Hovanec and Delong, (1996).

The community of ammonia oxidizers has been investigated in different environments and found to be more complex. Studying lake samples using a wide range of 16S rDNA primers showed a variation in species composition with depth and between aquatic environments according to differences in temperature, oxygen and inorganic nutrient concentrations (Ward et al., 1997). However, all these studies did not include sequence analysis and were done by genus specific primers or probes. Since *Nitrosococcus oceani* and *Nitrosococcus halophilus*, which belong to ? subdivision of the *Proteobacteria*, were isolated from marine environments, it was thought that they would be the dominant population in marine

environments. Although, *Proteobacteria* such as *Nitrosococcus oceani* were detected in seawater from the southern California Bight (Ward et al., 1982) and in several permanently ice-covered Antarctic saline lakes (Voytek et al., 1999), the dominant populations of marine sediments belonged to the β *Proteobacterial* ammonia oxidizers, while *Proteobacteria* such as *Nitrosococcus oceani* were minor part of this community (Nold et al., 2000) and could not be detected at all in the hypersaline Mono Lake, California (Ward et al., 2000). These results might have been biased due to the limited coverage of the specific primers used for PCR amplification. The dominant group in the Arctic Ocean (Bano and Hollibaugh, 2000), in marine sediments (Stephen et al., 1996), and in seaward dunes (Kowalchuk et al., 1997) was *Nitrospira* (cluster 1), while *Nitrosomonas* was also detected. *Nitrospira* cluster 1 was found mainly in marine environments, while other *Nitrospira* species could not be detected in these environments. Interestingly, Phillips et al. (1999) observed that members of *Nitrospira* (cluster 1) were dominated in planktonic samples while members of *Nitrosomonas* were dominated in particle-associated samples, showing that there was distinct separation by genus level in distinct niches of marine environments. It has long been known that at least some, if not all, of the ammonia oxidizers tend to adhere to surfaces (Prosser, 1989). The ability to form aggregates, to attach to soil, sediment suspended particles in water, or the surface of culture vessels is due to their capability to produce extracellular polymers (Stehr et al., 1995b). Exopolymers can serve as survival strategy during recovery after desiccation conditions in soil (Allison and Prosser, 1991a) and after starvation conditions (Abeliovich, 1987; Diab and Shilo, 1988), and facilitate nitrification at low pH (Allison and Prosser, 1993). The appearance of *Nitrosomonas* in particle-associated samples could also imply for different niches with different ammonia concentrations, while *Nitrosomonas* species prefer high ammonium concentrations. Interestingly, Speksnijder et al. (1998) were the first that could detect *Nitrosomonas* cluster 6 (including *Nitrosomonas ureae* and *Nitrosomonas oligotropha*), as dominant community of ammonia oxidizers in fresh water habitats. They could explain why this cluster could not be detected before in these habitats by using slight

modified primer sets. Bollmann and Laanbroek, (2001) supported the assumption that this cluster could grow under low concentrations of ammonium using continuous cultures medium containing 0.2 mM ammonium. Furthermore, a comparison of isolate G5-7, belongs to *Nitrosomonas* cluster 6, to *Nitrosomonas europaea*, belongs to *Nitrosomonas* cluster 7, showed that G5-7 grows better under low concentrations of ammonium but has a longer regeneration time after starvation (Bollmann et al., 2002). This implies niche differentiation. While G5-7 would be more prevalent at environments with continuously low ammonium concentrations, *Nitrosomonas europaea* would have an advantage in habitats with irregular pulses of ammonium. Another oligotrophic freshwater lake showed both *Nitrospira*-like bacteria and *Nitrosomonas* cluster 7, but *Nitrosomonas* cluster 7 could only be detected during the summer months when ammonium concentrations were increased (Whitby et al., 1999). This is in agreement with detection of this cluster in sewage treatments systems, (Mobarry et al., 1996) enrichments cultures, (Hiorns et al., 1995) and fertilized soils (Hastings et al., 1997), but is in contrast to the failure of their detection in eutrophic lakes (Hiorns et al., 1995; Hastings et al., 1998). Other environments with high ammonium concentrations are activated sludge, which are dominated by *Nitrosococcus mobilis*, (Juretschko et al., 1998; Rowan et al., in press). Altogether, there is some evidence that different ammonia concentrations in activated sludge, fresh water lakes and sediments have caused an adaptive evolution of different subgroups (Stehr et al., 1995a; Hastings et al., 1997; Suwa et al., 1997; Bollmann et al., 2002).

10. Community structure of ammonia oxidizers in different soils

The community structure of ammonia-oxidizing bacteria has been investigated in various soils that differ in environmental conditions such as pH, ammonium concentration, water content, or in cultivars of rice and in fertilizer management (Table 1). Most studies suggest that *Nitrospira* species of cluster 2, 3 and 4 are dominating in soils (Stephen et al., 1996; Kowalchuk et al., 1997; Stephen et al., 1998; Kowalchuk et al., 1998; Bruns et al., 1999;

Mendum et al., 1999; Phillips et al., 2000; Hastings et al., 2000; Kowalchuk et al., 2000a; 2000b). However, there is one study that shows the dominance of *Nitrosomonas* species in an acidic forest soil with and without liming (Carnol et al., 2002). Moreover, recently sequences of *Nitrospira* cluster 1, which were only found in marine environments in the past, were detected in acidic forest soils from Finland and Scotland (T. Aarnio, C.J. Phillips and J.I. Prosser, unpublished data; G.R. Ammpbell unpublished data as quoted by Webster et al., 2002). However, the abundance of *Nitrospira* cluster 1 within soil samples appeared to be less than in marine environments.

Table 1: Soil factors and composition of ammonia oxidizer community*

Environmental factor	Cluster by 16S rDNA/ <i>amo</i> A	Reference
Acidic pH	16S rDNA <i>Nitrospira</i> cluster 2	Stephen et al., 1996; 1998; Kowalchuk et al., 2000a
Acidic pH	16S rDNA <i>Nitrosomonas</i>	Carnol et al., 2002
High ammonium concentration or improved soils	16S rDNA <i>Nitrospira</i> cluster 3	Kowalchuk et al., 2000b
Low ammonium concentration	16S rDNA <i>Nitrospira</i> cluster 4 and 2	Kowalchuk et al., 2000b
Improved soils	16S rDNA <i>Nitrospira</i> clusters 1 and 3 and <i>Nitrosomonas</i> cluster 7	Webster et al., 2002
Unimproved soils	16S rDNA <i>Nitrospira</i> cluster 3 and <i>Nitrosomonas</i> cluster 7	Webster et al., 2002
Limitation of moisture	16S rDNA <i>Nitrosomonas</i> species exhibit more rapid recovery than <i>Nitrospira</i> species	Hastings et al., 2000
Soils irrigated with fertilizer-amended water	<i>amo</i> A <i>Nitrospira</i> -like (cluster 3 and 9)	Oved et al., 2001
Effluent-irrigated soils Improved agriculture soil	<i>amo</i> A <i>Nitrosomonas</i> -like <i>amo</i> A <i>Nitrospira</i> -like cluster 1, 3a, 3b, 4, 9)	Oved et al., 2001 Avrahami et al., 2002 (see appendix)

*ammonia oxidizers groups, which were dominant in the respective soils.

Study of the root environment in different rice cultivars under identical environment conditions showed high differentiation of community composition. While in one of the cultivars there was clear enrichment of *Nitrosomonas* spp. (40-69% of total ammonia oxidizers), *Nitrosomonas* was less abundant than the others (7-23% of total ammonia

oxidizers). Although in several studies of bulk soil nitrification activity showed high variability over space and time or in different fertilizer treatments, the community structure remained stable and only the population size increased (Phillips et al., 2000; Laverman et al., 2001). Comparison of five soil or sediment samples varying in oxygen availability showed no evidence of a particular group's being specific for anoxic environments (Kowalchuk et al., 1998). Furthermore, Smith et al. (2001) showed that growth conditions of enrichment cultures had no detectable effect on the affiliation of the enriched ammonia oxidizers to specific clusters. On the other hand, Hastings et al. (2000) observed that ammonia oxidizers decreased in number during limitation of moisture, and *Nitrosomonas* species exhibited more rapid recovery than *Nitrospira* species. Another selective factor in natural environments seems to be ammonium concentration. Kowalchuk et al. (2000a; 2000b) found that members of *Nitrospira* cluster 3 were dominant in early successional soils with relatively high ammonium concentration, while members of *Nitrospira* clusters 2 and 4 were dominant in old successional soils with low ammonium concentration. This was supported by enrichment cultures using different ammonium concentrations, which revealed biases toward *Nitrospira* cluster 3 or *Nitrospira* cluster 4 under high or low ammonium concentrations, respectively (Kowalchuk et al., 2000b). Moreover, comparison of native soils and tilled soils with different successional treatments showed that *Nitrospira* cluster 3 was mainly found in amended soils. DNA samples of amended soil could only be hybridized with a specific probe for *Nitrospira* cluster 3, while hybridization of DNA samples from native soil showed higher diversity and included *Nitrospira* cluster 2, 3 and 4 (Bruns et al., 1999; Webster et al., 2002). Phillips et al. (2000) studied different fertilizer treatments of agricultural soils and found *Nitrospira* cluster 3 in all treatments. Moreover, there appears to be some correlation between low soil pH and the dominance of *Nitrospira* cluster 2. Also, *Nitrospira* AP. AHB1, which was isolated with acidic medium, and several clones and enrichment cultures from acidic soils belong to *Nitrospira* cluster 2 (Stephen et al., 1996; 1998; Kowalchuk et al., 2000b; Laverman et al., 2001). There is one exception of an acidic forest soil, which

shows a dominance of *Nitrosomonas* species (Carnol et al., 2002). However, temperature as a selective environmental factor has not yet been tested.

11. Nitrifiers as trace gas producers

From the viewpoint of microbial trace gas metabolism, it is appropriate to distinguish between at least two different groups of soils: Upland soils, which are not water-saturated, are aerated and are generally oxic, and wetland soils, which are water-saturated soils that are generally anoxic. Uplands soils may contain anoxic niches (Tiedje et al., 1984; Sexstone et al., 1985; Zafiriou et al., 1989; Hoejberg et al., 1994). These two groups of soils have different characteristics with respect to microbial production and consumption of trace gases (Conrad, 1996). In all wetland soil studies the fluxes of N_2O were smaller compared to aerated soils, probably because a large percentage of the produced N_2O is further reduced by denitrifiers to N_2 (Conrad, 1996). Furthermore, submerged rice fields seem to act occasionally even as a sink for atmospheric N_2O (Minami and Fukushima, 1986). Therefore, it was more relevant studying uplands soil with respect to N_2O emissions.

Production and emission of N_2O into the environment is of major importance for global warming (Dickinson and Cicerone, 1986) as well as for the destruction of the stratospheric ozone layer (Crutzen, 1970). The estimated lifetime of N_2O is between 110 to 150 years (Williams et al., 1992). Nitrous oxide (N_2O) is produced as a by-product during nitrification (Poth and Focht, 1985) and as an intermediate during denitrification (Conrad, 1996). A method that is used to distinguish between denitrification to nitrification as a source of N_2O emission is based on the differential sensitivity of nitrification and denitrification to acetylene as an inhibitor. Using 10 Pa acetylene causes inhibition of nitrification but not denitrification, which is inhibited only by higher concentrations such as 10 kPa (Davidson et al., 1986; Robertson and Tiedje, 1987; Klemmedtsson et al., 1988). Production of nitrous oxide has also been demonstrated among bacteria that respire nitrate to nitrite and those that dissimilate nitrate to ammonium (DNRA) (Tiedje, 1988; Conrad, 1996). Nitrous oxide can be produced

chemically by decomposition of hydroxylamine, but this reaction seems to be of no or little importance under field conditions (Bremner et al., 1980; Minami and Fukushima, 1986).

Poth and Focht, (1985) and Poth, (1986) recognized that ammonia oxidizers could produce NO, N₂O and even N₂ under anaerobic conditions. However, N₂O was only produced in significant amounts after the culture was switched to anaerobic conditions. The shift to anaerobic conditions caused to acceleration in NO production as well (Remde and Conrad, 1990). More support of an increased production of N₂O due to stress is that under aerobic conditions *Nitrosomonas europaea* and *Nitrospira multiformis* produced higher amounts of N₂O at low pH or under substrate limitation (Jiang and Bakken, 1999). Furthermore, a decrease in temperature also caused an increase of N₂O/NO₃⁻ ratio at low temperatures. In other words, although nitrification rates were decreased, a relative larger proportion of N₂O was released from nitrification at low temperature (Maag and Vinther, 1996).

Microbial processes in soils contribute about 70% to the atmospheric budget of N₂O (Conrad, 1996). N₂O emissions from soils greatly increase with increasing N inputs by fertilization of agricultural soils (Skiba and Smith, 2000). Laboratory (Schuster and Conrad, 1992) and field studies (Müller et al., 1998) observed an increased contribution of nitrification to total N₂O production in correlation with increasing ammonium concentrations, thus indicating an increase in nitrifier activity after fertilization with ammonium. However, it remained unclear whether fertilization also results in a change in the community structure, as such a change was only observed for ammonia oxidizers when wastewater was applied instead of mineral fertilizer (Oved et al., 2001). Although field studies clearly indicate that the community structure of ammonia oxidizers can be different in different soils, few experimental studies exist which address the in-situ dynamics of ammonia oxidizer populations (Mendum et al., 1999; Phillips et al., 2000).

Several previous field studies demonstrated a positive correlation between temperature and N₂O emission rates (Conrad et al., 1983; Slemr et al., 1984; Williams and Fehsenfeld, 1991; Clayton et al., 1997; MacDonald et al., 1997; Smith et al., 1998; Mogge et al., 1998; Carnol

and Ineson, 1999; Mogge et al., 1999) G  dde and Conrad, (1999) studied the effect of temperature in two different set ups. In the first set up, the “temperature shift experiment”, the soil samples were incubated at 25  C followed by exposure to different temperatures, and showed a monotonous increase with increase in temperature. In the second set up, the “discrete temperature experiment”, the soil samples were adapted to different temperatures for five days, and inconsistently with the first laboratory experiment and previous field observations, higher production rates of NO and N₂O were observed at low (4  C) and high (37  C) than at intermediate (15  C or 25  C) temperatures. Such a pattern is in contrast to the monotonous increase in activity that is expected when a soil microbial community is exposed to increasing temperatures. Therefore, the authors speculated that the activity pattern might be due to the changes in the microbial community caused by the different incubation temperatures. Inhibition studies indicated that the N₂O-producing microflora consisted mostly of ammonia oxidizers (G  dde and Conrad, 1999). Hence, a specifically adapted ammonia oxidizer population might have developed within the relatively short period of 5 days.

Objectives of this study:

This study had three major parts.

1. The major objective of the first part was to optimize the amoA PCR system by testing non-degenerate primers. Similar to Nicolaisen and Ramsing, (2002) we observed multiple DGGE bands due to the two wobble positions in the amoA reverse primer, i.e. amoA-2R (Rotthauwe et al., 1997). It seems to be desirable to check with our samples whether non-degenerate amoA primers would give better results.
2. The second part focused on the effect of temperature and soil ammonium concentration on the activity and community structure of ammonia oxidizers. The major objectives of this part were:
 - To test how a change in soil ammonium concentration affects the activity and structure of the ammonium oxidizers after short-term incubation.
 - To find a conceivable explanation for the observation by G  dde and Conrad, (1999) that NO and N₂O production rates reached a maximum after short-term adaptation to intermediate temperatures. For this purpose we used soil taken from the same location.

These two experiments showed that a relatively short incubation (i.e. 4 weeks) of soil at different ammonium concentrations did not influence the community structure of ammonia oxidizers (Avrahami et al., 2002). Since ammonia oxidizers are known to be slow-growing bacteria, longer incubation may be necessary for detection of a community shift. Therefore, it cannot be ruled out that community changes occur after longer time periods and that these changes are influenced by soil temperature or other environmental conditions. Another objective of this part was:

- To demonstrate potential effects of temperature and fertilization on the composition of the soil ammonia oxidizer community after long-term incubation. Since temperature seems to influence the soil ammonium concentrations after a short incubation, we assumed that any effect of temperature would have to be tested in different ammonium

concentrations. In this part we focused on the same soil used in the short-term experiments.

3. The primary conclusion drawn from the previous part of the study was that the community shifts were clearly influenced by the different fertilizer treatments, indicating that ammonium was a selective factor for different ammonia oxidizer populations. However, temperature was also a selective factor, in particular since community shifts were also observed in the soil slurries, in which ammonium concentrations and pH were better controlled. Therefore, it was important to study the effect of temperature in more detail.

The main objective of the third part of the study was:

- To investigate the effect of temperature on community structure of ammonia oxidizers in three meadow soils with different diversity and mean annual temperature

II. Material and Methods

1. Material used in this study:

Chemicals

Source

40% Polyacrylamid/Bis solution 37.5:1	Bio Rad, München, Germany
Seakem [®] LE agarose	BMA BioWhittaker Molecular Applications, Rockland, ME, USA
Ammoniumperoxodisulfate (APS)	Bio Rad, München, Germany
Bromophenol blue	Fluka, Taufkirchen, Germany
Chloroform (pH 8)	Sigma Aldrich, Deisenhofen, Germany
EDTA	Roth, Karlsruhe, Germany
Formamide	Qbiogene, Heidelberg, Germany
Formamide loading dye	Amersham-Pharmacia Biotech, Freiburg, Germany
Isoamyl alcohol	Merck, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
Master Amp 2X PCR PremixE/F	Epicentre Technologies, Madison, WI, USA
PCR water	Sigma Aldrich, Deisenhofen, Germany
Phenol	Sigma Aldrich, Deisenhofen, Germany
Phenol Chloroform Isoamylalcohol 25:24:1 (pH 8)	Sigma Aldrich, Deisenhofen, Germany
Polyvinylpyrrolidone (PVPP)	Sigma Aldrich, Deisenhofen, Germany
Ready load ? DNA Hind III digested	Invitrogen, Groningen, The Netherlands
Sodium Dodecyl Sulfate (SDS)	Roth, Karlsruhe, Germany
SYBR Green I DNA staining	BMA BioWhittaker Molecular Applications, Rockland, ME, USA
TEMED	Bio Rad, München, Germany
50 X TAE buffer (50xTris-acetate-EDTA)	Bio Rad, München, Germany
Tris	Roth, Karlsruhe, Germany
Urea	Merck, Darmstadt, Germany

Enzymes

Source

Ampli <i>Taq</i> DNA polymerase	Perkin Elmer – Applied Biosystem, Weiterstadt, Germany
Lysozyme from chicken egg	Sigma Aldrich, Deisenhofen, Germany
Proteinase K	Sigma Aldrich, Deisenhofen, Germany

Kits

Fast DNA spin kit for soil
 Wizard[®] DNA Clean up
 QIAquick[®] gel extraction kit
 PGEM-T-Easy Vector system
 TA cloning kit
 QIAquick[™] PCR purification kit
 ABI Big Dye terminator kit version 2.0
 QIAquick[®] spin Miniprep kit

Source

Qbiogene, Heidelberg, Germany
 Promega, Madison, Wis. USA
 Qiagen, Hilden, Germany
 Promega, Madison, Wis. USA
 Invitrogen, Groningen, The Netherlands
 Qiagen, Hilden, Germany
 Perkin Elmer – Applied Biosystem, Weiterstadt, Germany
 Qiagen, Hilden, Germany

Other materials

Autoseq G-50 columns
 RC-membrane filters (0.2 µm)
 Celulose-acetate filters (0.2 µm)
 Filter paper 595 (1/2) Ø 210 mm
 Gel Bond PAG
 Glass beads (0.17- to 0.18-mm diameter)
 Escherichia coli JM109 competent cells
 Micro Bio-Spin[®] Chromatography column
 Autoseq G-50 columns

Source

Amersham-Pharmacia Biotech, Freiburg, Germany
 Schleicher & Schuell, Dassel, Germany
 Schleicher & Schuell, Dassel, Germany
 Schleicher & Schuell, Dassel, Germany
 BMA BioWhittaker Molecular Applications, Rockland, ME, USA
 Braun Biotech; Melsungen, Germany
 Promega, Madison, Wis. USA
 Bio Rad, München, Germany
 Amersham-Pharmacia Biotech, Freiburg, Germany

LB media

Ampicillin
 IPTG
 X-Gal

Source

Sigma Aldrich, Deisenhofen, Germany
 Sigma Aldrich, Deisenhofen, Germany
 Roth, Karlsruhe, Germany

2. Soil samples:

Soil samples were taken in April 2000 from an agricultural field near Eberstadt, Germany. The site (EAS) was the same used for previous studies (Gödde and Conrad, 1999). More soil samples were taken from three different meadow fields; KMS was sampled from a field close

to Moshav Kahal, Lake Kinneret (Israel), while GMS and OMS soils were sampled from fields near Giessen, and Oppenrod (Germany), respectively. For testing newly designed primers all the four soils mentioned above were used. The soils were different in their pH ranging between acidic (pH 5.0) and slightly alkaline (7.9). The mean annual temperature measured for the soils sampled in Germany was lower ($9.9 \pm 1.9^{\circ}\text{C}$, measured in Giessen) than that for the Israeli soil ($22.2 \pm 1.8^{\circ}\text{C}$). The main soils characteristics are given in Table 2. All soil samples were partly air-dried to 12.5 - 20.9% gravimetric water content, sieved to < 2 mm aggregate size and stored at 4°C .

Table 2: Characteristics of the soils

Soil	EAS soil Agriculture soil (Eberstadt, Germany)	GMS soil Meadow soil (Giessen, Germany)	OMS soil Meadow soil (Oppenrod, Germany)	KMS soil Meadow soil (Kahal, Israel)
Characteristics				
Ammonium [$\mu\text{g NH}_4^+\text{-N/g soil}$]	0.18	0.45	0.39	1.7
pH with water/ CaCl_2	6.1 / 5.8	5.78 / 4.94	5.0 / 4.7	7.93 / 7.4
60% WHC [g water/ 100g soil]	21.3	29.8	27.36	40.9
Mean annual temperature [$^{\circ}\text{C}$]	N.D.	$9.9 \pm 1.9^{\circ}\text{C}$ at 1999	N.D.	20.2 ± 1.8 at 2001
Soil's texture	Silt loam	Sandy loam over clay	Clay loam	N.D.
Carbon/Nitrogen [%]	1.69 / 0.16	3.66 / 0.31	2.7 / 0.21	3.75 / 0.37
Location	N50° 28.933', E8° 45.930'	N 50° 32', E 8° 47.3'	N50°35.022', E8°47.101'	Near Kinneret Lake
Sampling date	April 2000	February 2000	April 2000	November 2000

N.D. – not determined

3. Ammonium measurements

Measurements of ammonium followed by extraction of soil with 1 M KCl (1 g: 10 ml KCl) on a shaker (200 rpm) for 1 hour at 4°C , centrifugation for 10 minutes at 4°C and filtration (Celullose-acetate filters). Samples were stored at -20°C before analyzing calorimetrically (Kandeler and Gerber, 1988): Addition of solution I to the sample (1:1) and vortex was followed by addition of solution II (1:0.4; sample: diluted solution II). Measuring

of O.D. was performed by a spectrophotometer (Hitachi, U-110 spectrophotometer, Japan) after at least 30 minutes of incubation in the dark.

<u>Solution I</u>	g per 100 ml H ₂ O
Na-Salicylate	8.5
Na-Nitroprusside	0.06

<u>Solution II*</u>	g per 1 litter H ₂ O
Na-Dichloroisocyanurate	1

*Before the measurements this solution was diluted 1:3 with 0.3 M NaOH

4. Measurement of pH, gravimetric water and water holding capacity

The pH was determined after suspension of the soil in 0.01 M CaCl₂ or in water (Schlichting and Blume, 1966): Shaking of 10 g soil mixed with 25 ml CaCl₂ or water for 1 hour (200 rpm), followed by 1 hour of standing without shaking. Further shaking by hand followed by 10 minutes of centrifugation, and measuring the pH with an electrode (pH-meter, Schott Geräte GmbH, Germany). Soil gravimetric water content was measured by weighting the soils before and after over night incubation at 105°C. Maximal water holding capacity (WHC) was determined by standard protocol (Schlichting and Blume, 1966): A core of each soil covered with gauze, incubated in a water bath until the soil become saturated, followed by incubation for an hour in wet sand, weighting, overnight incubation in 105°C and again weighting. The water content of the soil was calculated and estimated as Maximal water holding capacity (WHC). At the beginning of each experiment the soils were adjusted to 60% WHC.

5. Experiment's set up

Activity measurements and community analysis of ammonia oxidizers were all done with all four soils. There were four different experimental set-ups, while only EAS soil was incubated in all of them and the only soil incubated for a short period.

5.1 Short-term incubations

For short-term incubations, EAS soil was amended with $70 \mu\text{g NH}_4^+\text{-N (gdw soil)}^{-1}$ followed by incubation for five days at different temperatures (4, 10, 15, 20, 25 and 37°C). The experimental set-up was the same as that used by Gødde and Conrad (1999) for the “discrete temperature experiment” except for the addition of ammonium. After 5 days of incubation ammonium concentrations, release rates of N_2O , and contribution of nitrification to N_2O release were measured. N_2O was analyzed in a Carlo Erba 8000 gas chromatograph (GC) equipped with an electron capture detector (Gødde and Conrad, 1999). Contribution of nitrification to N_2O release was measured after preincubation with and without 10 Pa acetylene (Gødde and Conrad, 1999). This set up also used to investigate the effect of ammonium concentration of the soil, while ammonium content was adjusted to three different concentrations, i.e. 6.5, 58, and $395 \mu\text{g NH}_4^+\text{-N (gdw soil)}^{-1}$, followed by incubation at 4°C (Avrahami et al., 2002) (see appendix).

5.2. Long-term incubations

For long-term incubations, all soils were amended with a commercial fertilizer (FLATANIA, terrasan) with a total N concentration of 13% (w/w). The fertilizer consisted mainly (70%) of horn and hoof material, thus allowing for slow release of N. KMS soil was amended with 0.5-1.5% of fertilizer at two time periods during incubation (Table 3). EAS and OMS soils were amended at the beginning with two concentrations of fertilizer, high fertilizer (HF) treatment (1% w/w) and low fertilizer (LF) treatment (0.3% w/w). GMS soil received only a HF treatment. HF treatments (EAS, GMS and OMS) were set up in 120-ml bottles with

5 g of moist soil, while HF of KMS soil and LF treatments (EAS and OMS) were set up in 500-ml boxes with ca 150 g of soil. The fertilizer was mixed into the moist soil. Both bottles and boxes were opened for aeration every three days for 10 minutes to maintain aerobic condition. Bottles and boxes were incubated at 4 to 37°C (Table 4). All incubations and samplings were done in duplicates. Boxes were weighted before incubation and every three days during incubation. Since we used non-tightly closed boxes, water was added occasionally, to compensate evaporation loss. The bottles were closed with parafilm to avoid loss of water after 6.5 weeks of incubation. The gravimetric water content of the soil samples was measured after 20 weeks of incubation. On the average, soil moisture had decreased from initially 60% WHC to $38 \pm 4\%$, $38 \pm 5\%$, and $42 \pm 7\%$ WHC (EAS, GMS and OMS, respectively).

Table 3: Percentage of fertilizer added to KMS at different temperatures

Temperature [°C]	Fertilizer addition At zero time + after 11 weeks [%]
4	0.5 + 0
10	0.5 + 1
15	1 + 1.5
20	1 + 1.5
25	1 + 1.5
30	0.5 + 1.5
37	0.1 + 0.5

Table 4: Temperatures of incubation for each treatment

Soil	LF treatment [°C]	HF treatment [°C]
EAS	4, 10, 15, 20, 25, 30, 37	4, 10, 15, 20, 25, 37
OMS	10, 15, 20, 25, 30	4, 25, 30
GMS	-	4, 25, 30
KMS	-	4, 10, 15, 20, 25, 30, 37

Ammonium was measured at zero time and after 6.5 weeks (HF treatments of EAS, OMS and GMS) or after 8, 16 and 20 weeks incubation (HF treatment of KMS and LF treatment of OMS) or after 16 and 20 weeks of incubation (LF treatment of EAS). Potential nitrification activities of all soils were measured after 20 weeks of incubation and in addition in LF treatment of EAS soil after 16 week of incubation using a modified protocol of Stienstra et al. (1994) and Schmidt and Belser, (1994). Sterile Erlenmeyer flasks containing 18 ml phosphate buffer (1 mM, pH 7.4), 0.04 ml $(\text{NH}_4)_2\text{SO}_4$ (0.25M), and 2 g of soil were incubated at 25°C on a shaker for 6 hours (HF treatment of EAS, GMS and OMS) or 38 hours (HF treatment of KMS and both LF treatments). Samples were taken in 5 intervals, centrifuged for 5 minutes (at 4°C) and filtered through RC-membrane filters (0.2 μm ; Schleicher & Schuell). Samples were stored at -20°C until analysis of nitrite and nitrate in a Sykam Ion Chromatograph system (Bak et al., 1991). No nitrite was detected. Rates of potential nitrification activity were determined from the slope of a linear regression of nitrate production versus time. Samples for community analysis were taken after 6.5 and 20 weeks of incubation (HF treatment of EAS, GMS and OMS), after 8, 16 and 20 weeks of incubation (HF treatment of KMS and LF treatment of OMS) or after 16 and 20 weeks of incubation (LF treatment of EAS).

Long-term incubations were also done using slurries of all soils. Slurries with 5 g of soil and 15 ml of mineral medium (MacDonald and Spokes, 1980 modified by Aakra et al. 1999b) were set up in 250-ml Erlenmeyer flasks. The medium contained nitrogen as urea at a concentration of about 4 mM. The pH was adjusted to 7.0-7.5 with 1 M NaOH once a week. Urea solution (4 mM) was added occasionally during the incubation in total amounts of 10-55 ml. The slurries were incubated in duplicate at 4, 10, 15, 20, 25, and 30°C. Samples for community analysis were taken after 5.5, 12.5 and 19.5 weeks of incubation.

<u>Mineral medium</u>	<u>Full name</u>	<u>Concentration</u>
Urea		3.78 mM
KH ₂ PO ₄	Potassium dihydrogen phosphate	1.47 mM
CaCl ₂ ·2H ₂ O	Calcium chloride dihydrate	140 µM
MgSO ₄ ·7H ₂ O	Magnesium sulfate heptahydrate	160 µM
HEPES	N-(2-hydroxyethyl)-piperazine -N'-2-ethanesulfonic acid	20 mM
Trace element solution		1 ml : 1 liter

Media was prepared with double distilled water filtered (0.2 µm) and sterilized by autoclaving (20 min, 121°C).

The pH was adjusted to 7.5 with 10 M NaOH before sterilization.

<u>Trace element solution</u>	<u>Full name</u>	<u>mg/ liter</u>	<u>Concentration</u>
FeNaEDTA	Ferric sodium EDTA	3.8	
NaMoO ₄ ·2H ₂ O	Sodium molybdate dihydrate	0.1	457 nM
MnCl ₂	Manganese chloride	0.2	1.23 µM
CoCl ₂ ·6H ₂ O	Cobaltous chloride hexahydrate	0.002	8.4 nM
ZnSO ₄ ·7H ₂ O	Zinc sulfate heptahydrate	0.1	348 nM
CuSO ₄ ·5H ₂ O	Cupric sulfate pentahydrate	0.02	80 nM
phenol red		1	
NaHCO ₃ *	Sodium hydrogen carbonate		1.5 mM

*addition after sterilization

6. Molecular analysis

6.1. DNA Extraction

All set-ups of short-term and long-term incubations were sampled for molecular analysis. Approximately 500 mg (wet weight) of soil were transferred into a 2-ml screw cup tube. Slurry samples (2 ml) were centrifuged at 4°C and the supernatant was removed.

DNA was extracted from samples of short-term incubation at different ammonium concentrations following the protocol of Lüdemann et al. (2000) with slight modifications. Each sample was mixed with 750 µl of Na-phosphate buffer (120 mM, pH 8) and 187 µl SDS-solution (10% SDS; 0.5 M Tris/HCl, pH 8; 0.1 M NaCl). The samples were re-

suspended homogenously by vortexing. After incubation for 10 min at 60°C, 0.5 g of glass beads (0.17-0.18 mm diameter) was added, and the suspension was shaken for one minute at maximum speed in a bead beater (Dismembrator-S, B. Braun Biotech; Melsungen, Germany). After centrifugation (10 min, 14,000 rpm at 4°C) the supernatant was collected and extracted three times with phenol (pH 8), phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) and chloroform:isoamyl alcohol (25:24). Addition of ammonium acetate (to a final concentration of 2.5 M), and an equal volume of cold isopropanol, followed by 30 min at -20°C and centrifugation (20 min at 4°C) allow precipitation of DNA. Subsequently, the DNA pellet was washed with 80% ethanol, centrifuged (5 min, 14,000 rpm at 4°C) and dried under vacuum. Finally, the DNA was re-suspended with 100 µl of TE buffer (10 mM Tris base, 1 mM EDTA, pH 8). DNA was cleaned from humic acids, which were removed with acid-washed polyvinyl-polypyrrolidone (PVPP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in spin columns (Bio-Rad, Munich, Germany) (Henckel et al., 2000): The spin columns were filled with 2 ml of PVPP, which had been equilibrated and suspended in Tris-EDTA (pH 8). The PVPP columns were packed and dried by centrifugation ($375 \times g$ for 1 min) just prior to loading. About 150 µl of the brownish humic acid-containing soil DNA extract was loaded onto the column and centrifuged. The purified DNA solution was clear, colorless and readily amplifiable by PCR.

DNA of other samples: long-term experiments in moist soil and in slurry sets-up were extracted using the Fast DNA[®] SPIN[®] Kit for Soil (BIO 101, Carlsbad, CA, USA), in accordance to the manufacturer's instructions. DNA was cleaned from humic acid, if necessary, using the Wizard DNA clean up kit (Promega, Madison, Wis. USA).

6.2. Test of primers

The non-degenerate amoA-2R primer was compared with 4 newly designed non-degenerate primers (amoAR1 to amoAR4, Table 5) using 8 *amoA* clones and DNA amplified from 4 environmental samples. The length of the PCR-amplified *amoA* fragments was 491

bp. PCR products were compared by DGGE (see below; gradient of 33-67%). In addition, a clone library was established from environmental samples using the primer set amoA2F/amoA5R (Webster et al., 2002) (Table 5). This resulted in longer *amoA* sequences (800 bp). The thermal profile used for amplification was: 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 48°C and 1 min at 72°C, and 7 min at 48°C for the last cycle. Clones (44) were partly sequenced and seven representative clones, which were fully sequenced, were used to generate plasmid mixtures. In addition, three pure cultures (*Nitrosomonas europaea* (ATCC 25978), *Nitrosomonas eutropha* and *Nitrospira multiformis* (ATCC 25196), supplied by P. Witzel (Plön), originally from H.P. Koops (Hamburg)) were amplified with amoA2F/amoA5R primers set and cloned. These clones were fully sequenced. While the sequence of *Nitrosomonas europaea* was identical to accession number L08050 (McTavish et al., 1993a), that of *Nitrosomonas eutropha* was different in 3 bp from U51630 (Norton et al., 2002), and that of *Nitrospira multiformis* was different in 3 bp from U15733. These bacterial clones were also used for plasmid mixtures. Plasmids were extracted using the QIAprep® Spin Miniprep kit (Qiagen, Hilden, Germany). Plasmid mixtures were amplified with the different primers, and PCR products analyzed by DGGE. In this experiment, an additional primer, amoA-Ino with inosine in two wobble positions, was used, similarly as described by Vainio and Hantula, (2000).

6.3. PCR amplification of *amoA*

The primers used for PCR amplification were the amoA-1F (Rotthauwe et al., 1997) and its modified amoA reverse primer (amoAR1, Table 5) or amoA-2R for the short-term experiment. For DGGE analysis a GC clamp (Muyzer et al., 1997) was added to the 5' end of amoA-1F primer. Amplification was performed by using 0.5 µM of each primer, 1 unit AmpliTaq DNA polymerase (Perkin- Elmer Applied Biosystems, Weiterstadt, Germany), and 25µl of MasterAmpTM 2xPCR premix F and E (for amoA-2R and amoAR1, respectively) containing 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 5 mM MgCl₂, 400µM of each

deoxynucleoside triphosphate, and PCR enhancer betaine (Epicentre Technologies, Madison, WI, USA). DNA (0.5 -1 µl) was added to a final volume of 50 µl. If necessary, DNA was diluted 10 times and 1 µl used for amplification. Amplifications always started by placing PCR tubes into the preheated (94°C) thermal block of a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Thermal profile used for amplification of short fragments was modified after Rothauwe et al. (1997) and included 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 57°C and 1 min at 72°C, and 7 min at 72°C for the last cycle. For amoA-2R annealing temperature was 60°C and for the plasmid mixtures only 20 cycles were applied.

Table 5: Primer's used in this study

Name of Primer	Sequence of primer	Position*	Reference
amoA-2R	CCC CTC KGS AAA GCC TTC TTC	802 - 822	Rothauwe et al., 1997
amoAR1	CCC CTC GGG AAA GCC TTC TTC	802	This study
amoAR2	CCC CTC GGC AAA GCC TTC TTC	802	This study
amoAR3	CCC CTC TGG AAA GCC TTC TTC	802	This study
amoAR4	CCC CTC TGC AAA GCC TTC TTC	802	This study
amoA-Ino	CCC CTC IGI AAA GCC TTC TTC	802	This study
amoA-1F**	GGG GTT TCT ACT GGT GGT	332 - 349	Rothauwe et al., 1997
amoA-2F	AAR GCG GCS AAG ATG CCG CC	279 – 298	Webster et al., 2002
amoA-5R	TTA TTT GAT CCC CT	1065 - 1079	Webster et al., 2002

* according to the open reading frame published previously for the *amo A* gene sequence of *Nitrosomonas europaea* (McTavish et al., 1993a).

** with addition of GC clamp (Muyzer et al., 1997) : CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G

6.4. DGGE and cloning

DGGE was preformed as described previously, with slight modifications (Muyzer et al., 1997). PCR products were separated on a polyacrylamide gel, using a gradient of 45% (6% [wt/vol] acrylamide-bisacrylamide [37.5:1]; Bio-Rad (Labratories, GmbH, Munich, Germany), 18% deionized formamide, 3.1 M urea) to 65% (6% [wt/vol] acrylamide-bisacrylamide [37.5:1]; Bio-Rad, 26% deionized formamide, 4.5 M urea). If necessary, the

gradient was changed to a narrower gradient, which gives a better resolution. Gels were electrophoresed by using the D GENETM system (Bio Rad) with 0.5xTris-acetate-EDTA at 60°C at 100 V for 17 h. Gels were stained with SYBR Green (BMA BioWhittaker Molecular Applications, Rockland, ME, USA) and scanned with a Storm 860 phosphor imager (Molecular Dynamics, Sunnyvale, Calif. USA).

DGGE stock solutions:

0% Urea-Formamide solution

40% Polyacrylamid/Bis stock solution	75 ml
50 X TAE	10 ml
Double distilled water filtered (0.2 µm)	Fill to 500 ml

80% Urea-Formamide solution

40% Polyacrylamid/Bis stock solution	75 ml
50 X TAE	10 ml
Urea	168 g
Formamide (deionized)	160 ml
Double distilled water filtered (0.2 µm)	Fill to 500 ml

Bands were excised from DGGE using a Dark Reader transilluminator (Clare Chemical research, Ross on Wye, UK). The excised bands were suspended into 200 µl of PCR water, re-amplified and electrophoresed on DGGE again. Some bands were repeatedly electrophoresed and excised until only one band was detectable on DGGE. The purified bands were sequenced. However, in many cases there were still multiple bands after several cycles of excision and re-amplification. Therefore, multiple bands excised, were re-excised, amplified, ligated to the pGEM Teasy vector, and were used to transform *Escherichia coli* JM109 competent cells (Promega, Madison, Wis.). Alternatively, re-amplified bands were cloned using the original TOPO cloning kit (pCR 2.1 vector for *Escherichia coli*: TOP 10F;

Invitrogen, Leek, The Netherlands) following the manufacturer's instructions. In some cases experimental samples were cloned. Clones containing a correct insert were re-amplified using *amoA* primers and screened by DGGE, always compared with their environmental/experimental sample. Different clone types re-amplified with *amoA* primers were sequenced as described below.

6.5. Sequencing

PCR products from clones were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Both strands were sequenced directly from 70 ng of PCR product with vector specific primers and the ABI BIG Dye terminator kit V2.0 (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. Subsequently, excess primers and dye terminators were removed with Autoseq G-50 columns (Amersham-Pharmacia Biotech, Freiburg, Germany) and cycle sequencing reactions were analyzed with an ABI 377 DNA sequencer (Applied Biosystems).

6.6. Phylogenetic analysis

Based on the sequence information deposited either in public-domain databases or generated in the course of this study, we established a database of *amoA*. This database was integrated into the ARB program package (<http://www.arb-home.de>). Derived *AmoA* sequence types with less than 99% similarity were taken for phylogenetic analysis. For sequence types, which exhibited at least 99% similarity to each other, only one representative was considered for construction of trees. Phylogenetic analyses were performed, based on 150 deduced amino acid positions, using ARB and the Phylip software package version 3.6a2.1 (Felsenstein, 1993). Trees were reconstructed using the PAM matrix in combination with neighbor-joining method (ARB and PHYLIP) or FITCH (PHYLIP) (Rotthauwe et al., 1997), parsimony PROTPARS (PHYLIP) or Maximum likelihood (PROTML) (Institute Pasteur, Paris; <http://bioweb.pasteur.fr/sequanal/interfaces/molphy.html>).

6.7. Nucleotide sequence accession numbers available

Sequences of *amoA* gene have been deposited in the GeneBank nucleotide sequence database under accession no. AY098867 through AY098890 (short period incubation of EAS soil or after field sampling); AY177925 through AY177933 (clones of 800 bp fragments); AY177934 through AY177938 (clones for comparison of the primers); AY177392, AY177394, AY177939 through AY177941, AY177946, AY177953 (clones or bands retrieved from high fertilizer (HF) treatment of EAS soil); AY177942, AY177948, AY177950, AY177954 through AY177966 (clones or bands retrieved from low fertilizer (LF) treatment of EAS soil); AY177943 through AY177945, AY177947, AY177949, AY177951, AY177952, AY177967 (clones or bands retrieved from slurry treatment of EAS soil). The rest of the sequences were not submitted to the GeneBank before the submission of this thesis.

6.8. Correspondence analysis

Correspondence analysis was performed on the data using SYSTAT 9 (SPSS Inc, Chicago, IL). Correspondence analysis is similar to principle component analysis, but is preferable for analysis of species abundance data if many zero values are present (Legendre and Legendre, 1998). The analysis compares two sets of descriptors (DGGE bands versus samples) using chi-square distances, and reduces the multidimensional relationships between them to two principle axes. In our data, the ordination of DGGE bands is used to predict the ordination of samples. That is, the DGGE bands are considered as descriptors that create the two-dimensional space in which the samples are scattered.

Chapter 1:

Optimizing the *amoA* PCR system to study the community structure of ammonia oxidizers using denaturing gradient gel electrophoresis (DGGE)

Results

Design of non-degenerate *amoA* primers

The *amoA* primer system published previously by Rotthauwe et al. (1997) was modified by separation of the degenerate reverse primer *amoA*-2R into 4 non-degenerate reverse primers (Table 5). The performance of the new primers was compared to the original degenerate one (*amoA*-2R) by using *amoA* clone sequences retrieved from different soils and representing different *Nitrosospira* clusters (Fig. 3). While the clone sequences showed only one DGGE band when using one of the non-degenerate primers, they exhibited up to four DGGE bands when using the degenerate primer *amoA*-2R. However, in most cases the DGGE band obtained with *amoA*R1 was similar to the dominant band obtained with *amoA*-2R.

Comparison of the primers using *amoA* PCR products from soil DNA showed fainter and less complex DGGE patterns with *amoA*-2R and *amoA*R4 than with one of the three other primers (Fig. 4), indicating that DGGE using *amoA*R1, *amoA*R2 and *amoA*R3 reproduced diversity of *amoA* sequences in soil better than using *amoA*-2R or *amoA*R4.

An *amoA* clone library was established from EAS soil and from GMS soil using the *amoA*2F/*amoA*5R primer system (Webster et al., 2002). This primer set amplified an *amoA* fragment of 800 bp length. The sequences of these long *amoA* fragments (44 clones) fell into different clusters of the genus *Nitrosospira*. Representative clones of these different *Nitrosospira* clusters showed variations in the target nucleotide positions according to the primer sequence position 7 and 9 of *amoA*-2R (Fig. 5; Table 6).

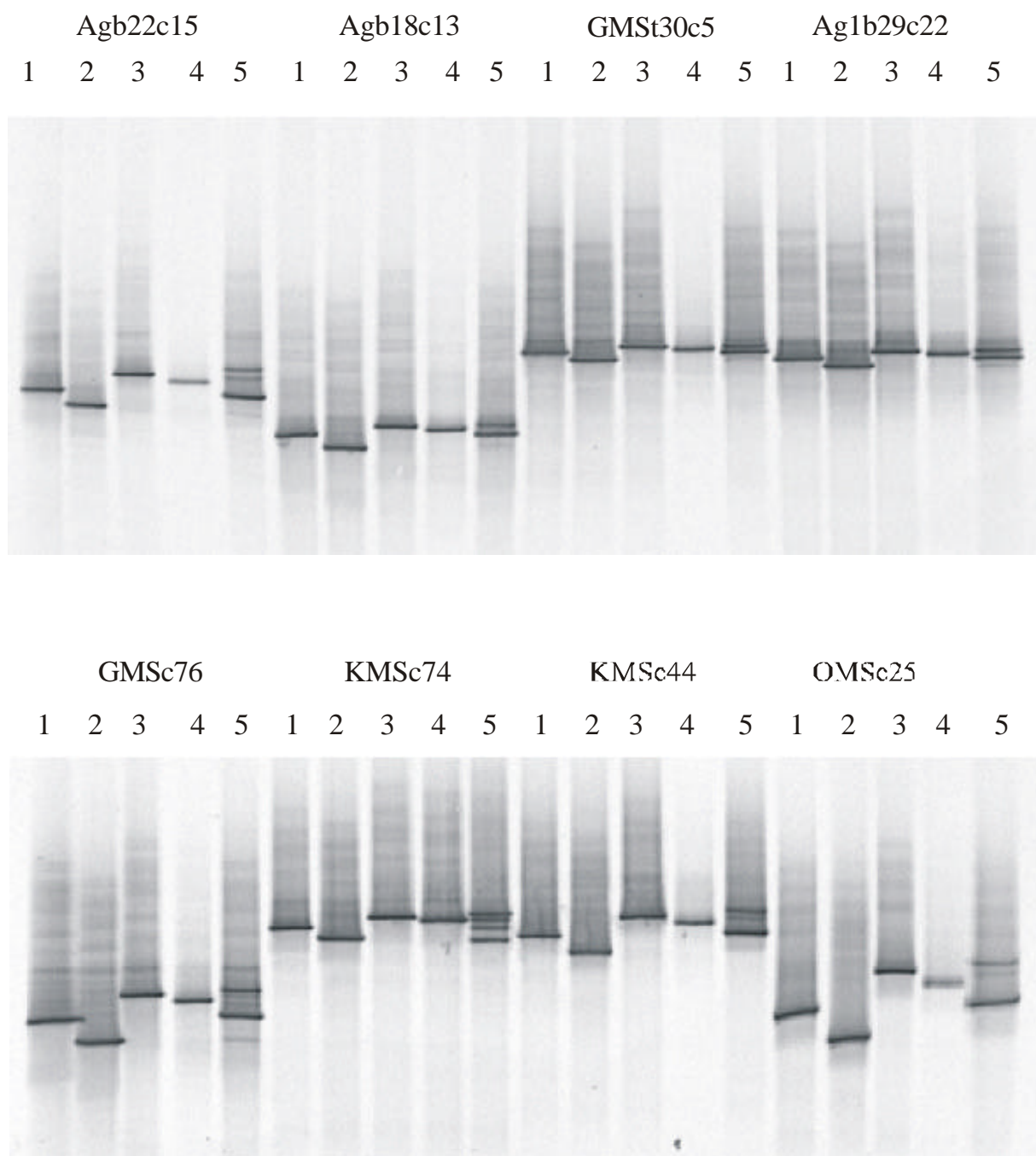


Figure 3: Comparison of DGGE banding patterns obtained by using five different reverse primers: 1- amoAR1, 2 - amoAR2, 3 - amoAR3, 4 - amoAR4 and 5 - amoA-2R. Using a DGGE gradient of 45 to 62 %, a mixture of the following eight *amoA* clones was analyzed: Agb22c15 (Acc. No. AY098879), Agb18c13 (Acc. No. AY098876), GMSt30c5 (Acc. No. AY177934), Agb29c22 (Acc. No. AY098882), Ms1c76 (Acc. No. AY177935), KMSc74 (Acc. No. AY177938), KMSc44 (Acc. No. AY177937), Ms2c25 (Acc. No. AY177936).

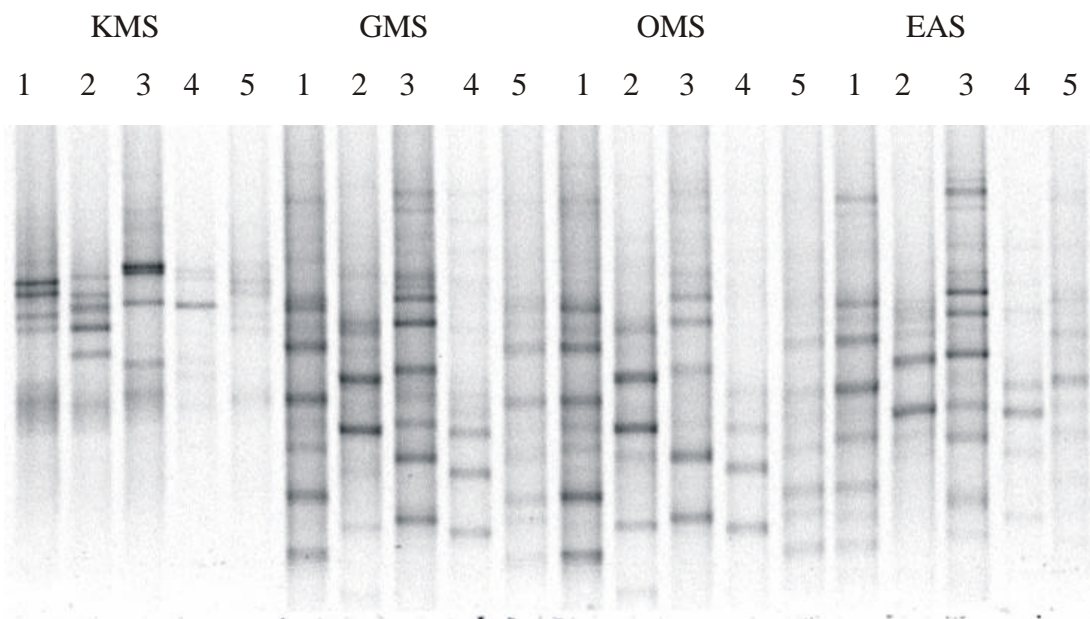


Figure 4: Comparison of DGGE banding patterns obtained by using five different reverse primers: 1- amoAR1, 2 - amoAR2, 3 - amoA3, 4 - amoA4 and 5 - amoA-2R. The following environmental samples were investigated using a DGGE gradient of 50 to 62%: KMS - Kahal meadow soil, GMS - Giessen meadow soil, OMS - Oppenrod meadow soil, EAS - Eberstadt agriculture soil (see table 2).

Table 6: Sequence of amoA-2R region retrieved from representative long fragments of *amo A* clones

Clone's name	Sequence of primer amoA-2R and corresponding clone sequences (target)
amoA-2R	CCC CTC KGS AAA GCC TTC TTC
sIEASLc7	CCC CTC GGG AAA GCC TTC TTG
sIEASLc25	CCC CTC GGG AAA GCC TTC TTG
slGMSLc3	CCC CTC CGG AAA CCC TTC TTG
sIEASLc44	CCC CTC CGG AAA CCC TTC TTC
Mul4	CCC CTC GGC AAA GCC TTC TTC
sIEASLc41a	CCC CTC GGC AAA GCC TTC TTC
sIEASLc18	CCC CTC CGG AAA CCG TTC TTG
sIEASLc41	CCC CTC CGG AAA CCC TTC TTC
Eur5	CCC CTC TGG AAA GCC TTC TTC
Eut1	CCC CTC TGG AAA GCC TTC TTC
	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21

Clones are highlighted (pink) in Fig. 5

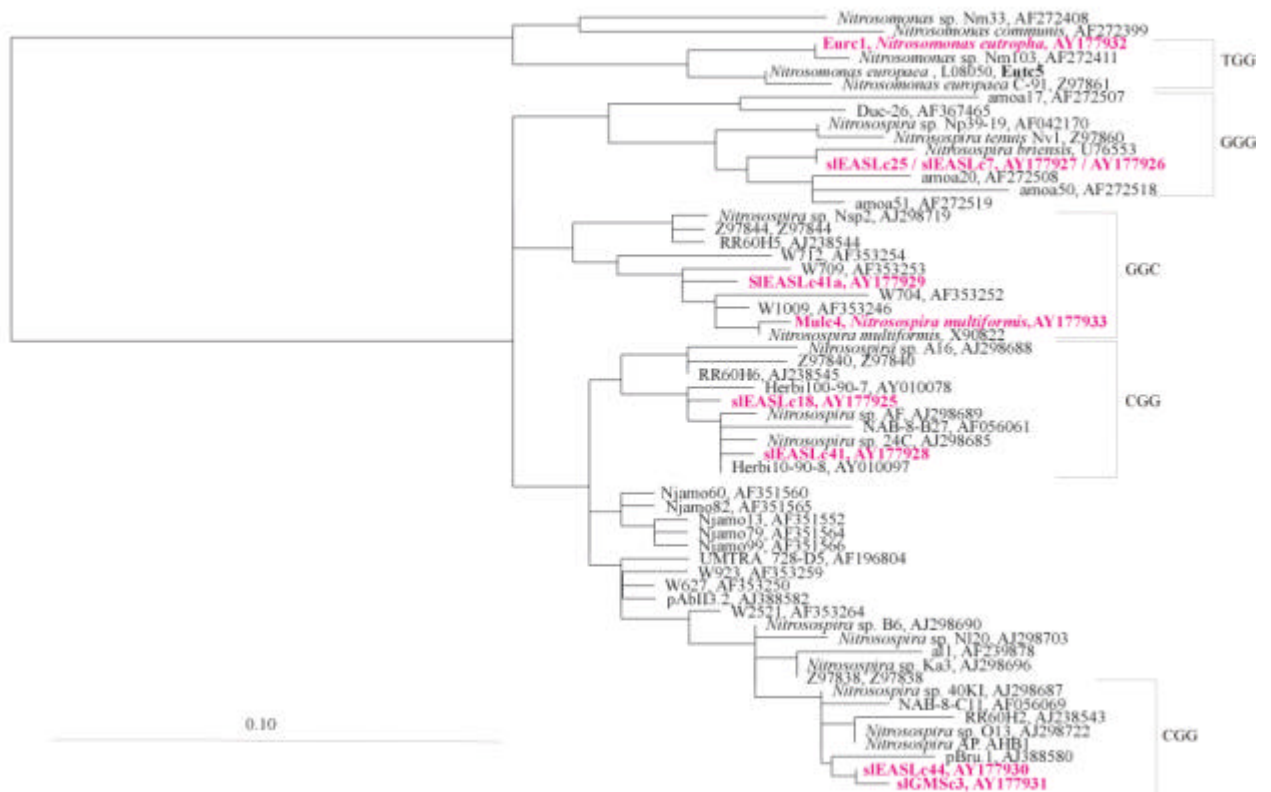


Figure 5: Maximum likelihood tree based on partial AmoA sequences (150 derived amino acid positions). Clone sequences obtained from this experiment are highlighted in bold pink. The three-nucleotide positions that correspond to all sequence types of the respective cluster represent the target sequence motif of primer amoA-2R as highlighted (red) in Table 6. Scale bar indicates 10 changes per 100 nucleotide positions.

Five different mixtures of the representative clones were amplified and analyzed by DGGE using the non-degenerate primers amoAR1 and amoAR3, as well as the original degenerate primer amoA-2R and a newly designed degenerate primer amoA-Ino that contained inosine in the target nucleotide positions 7 and 9. Consequently, amoA-Ino should have matched to all clone sequences (Table 6). The non-degenerate primers detected the clone sequences as well as the degenerate primers. However, none of the primers was able to detect sIEASLc18 and sIEASLc41 within the clone mixtures (Fig. 6), although one of the clones (sIEASLc41) was easily detectable by amoA-2R and amoAR1 when tested as a single clean plasmid or clone.

The clones sEASLc18 and sEASLc41 clustered with published *amoA* sequences related to ‘Herbi’ or ‘NAB’ and to *Nitrosospira* sp. 24C, respectively (Fig. 5). Sequences of these clones showed between one to three additional mismatches with the amoA-2R primer (Table 6).

Since DGGE patterns obtained with amoAR1 gave the dominant band seen in the DGGE obtained with degenerate amoA-2R (Fig. 3), exhibited a similar high diversity (Fig. 4) and detected the same number of clones in a mixture (Fig. 6), amoAR1 was chosen for further analysis of the experimental soil samples.

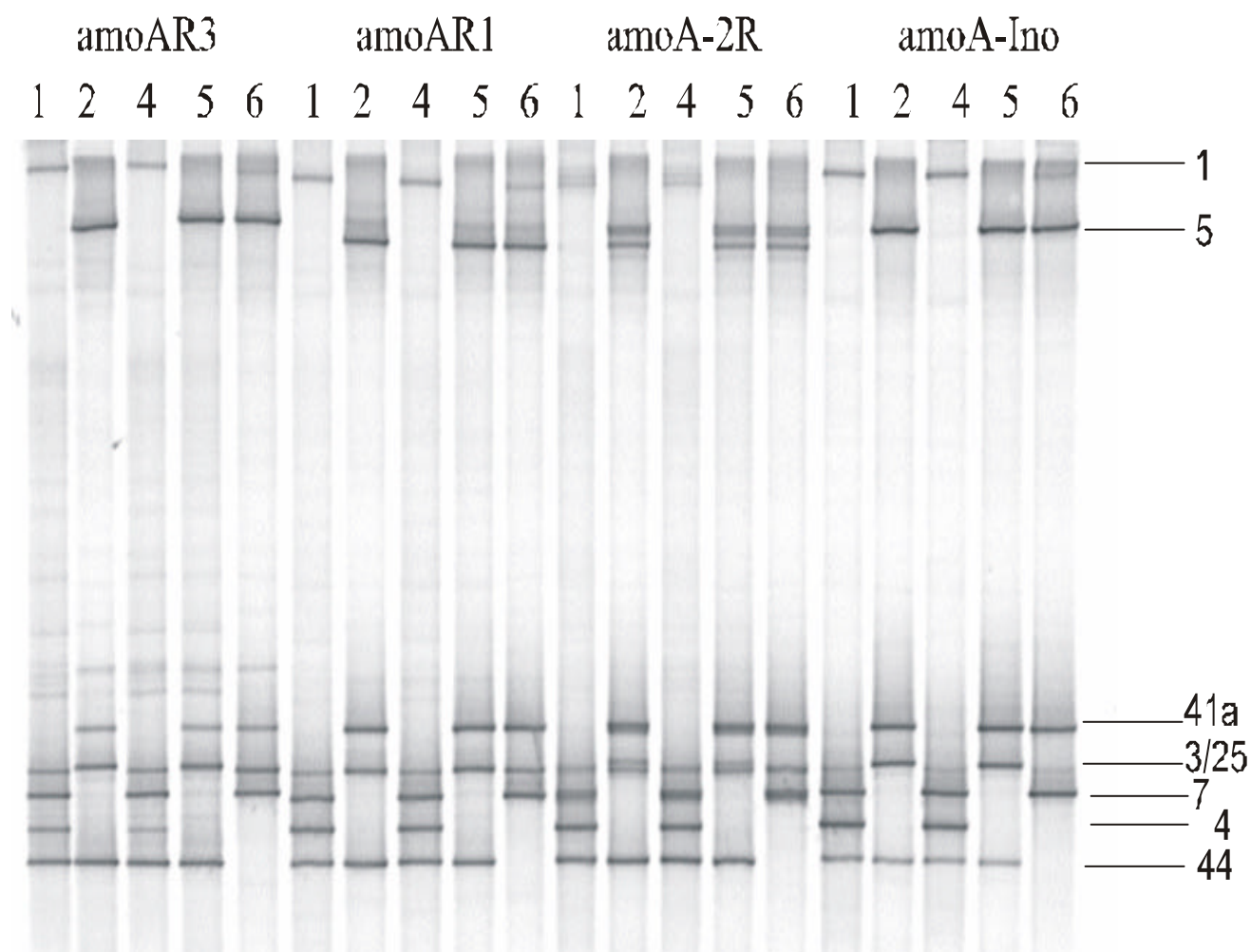


Figure 6 (previous page): Comparison of DGGE banding patterns obtained by using four different reverse primers: amoAR3, amoAR1, amoA-2R, and amoA-Ino. Using a DGGE gradient of 33 to 67%, the following five clone mixtures were assessed (numbers in the margin give the last digit instead of the full names of the clones below):

- 1: sIEASLc7, sIEASLc18, sIEASLc44, sIGMSLc3, Mulc4, Eurc1.
- 2: sIEASLc18, sIEASLc25, sIEASLc41, sIEASLc41a, sIEASLc44, Eutc5.
- 4: sIEASLc7, sIEASLc41, sIEASLc44, sIGMSLc3, Mulc4, Eurc1.
- 5: sIEASLc25, sIEASLc41, sIEASLc41a, sIEASLc44, sIGMSLc3, Eutc5.
- 6: sIEASLc7, sIEASLc41, sIEASLc41a, sIGMSLc3, Eutc5, Eurc1.

Discussion

The community structure of ammonia oxidizers in the different experiments was determined targeting *amoA* by using the PCR assay of Rotthauwe et al. (1997). This assay has been used before for community analysis (Rotthauwe et al., 1997; Oved et al., 2001; Nicolaisen and Ramsing, 2002). Oved et al. (2001) used the published *amoA* primers (with a degenerate reverse primer) for DGGE analysis of ammonia oxidizers in soils without problems by obtaining multiple bands for a single *amoA* clone. They used capillary PCR. This technique led to an efficient binding of the primers. Nicolaisen and Ramsing (2002), on the other hand, recognized multiple bands, and therefore decided to use the non-degenerate reverse primer *amoA*-2R-TC (synonymous to *amoAR4* in this work). The results of our experiments indicated that a different degenerate primer, i.e. *amoAR1*, was preferable for our soil systems, since it detected a higher diversity of *amoA* sequences than primer *amoA*-2R-TC. Our different experience may be due to the different communities of ammonia oxidizers in our soils (mainly *Nitrosospira* species) compared to the samples of Nicolaisen and Ramsing, (2002) containing mainly *Nitrososomonas* species. In the present study we decided to use the non-degenerate primer *amoAR1*, since it detected a relatively high diversity of *amoA* sequences in environmental samples. Nevertheless, our primer combination was unable to detect *amoA* sequences related to *Nitrosospira* sp. 24C (*Nitrosospira* cluster 10) if mixed with other sequences (Fig. 5). Indeed, no sequence from this cluster was detected in our DGGE-based community analyses. We cannot rule out that our inability to detect such sequences was due to the primer system used. Since the short-term experiment was done before the optimization of the primers, *amoA*-2R was used for analyzing of the communities of EAS soil after field sampling and short-term incubation. However, different bands represented different sequences and therefore there was no overestimation of the community.

Chapter 2:

**Effects of soil ammonium concentration, temperature, and
fertilizer on activity and community structure of soil
ammonia oxidizers**

Results

The agriculture soil, Eberstadt (EAS soil) was incubated in different ways :

1. Short-term effect of ammonium soil concentration on N₂O release

Rates of N₂O release were measured after five days of incubation at 4°C as a function of the initial ammonium concentration, i.e. low, medium and high (6.5, 58 and 395 µg NH₄⁺-N gdw soil⁻¹). The N₂O release rates increased with the initial ammonium concentration (Fig. 7). Final nitrate concentrations in the low, medium and high ammonium treatments were 18, 20 and 21 µg NO₃⁻-N gdw soil⁻¹, and were significantly different only between the low and high ammonium treatments (p<0.05). The contribution of nitrification to the total N₂O release shifted from 25% at low to 50 and 52% in the treatments with medium and high ammonium, respectively (Fig. 7).

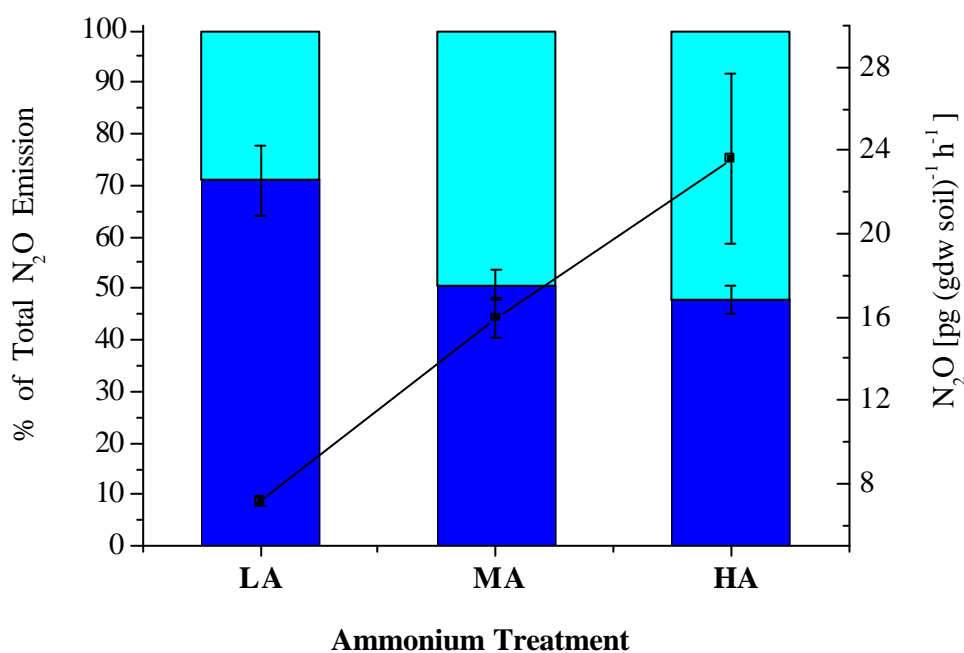


Figure 7: Effect of incubation at low (LA), medium (MA), and high (HA) ammonium concentrations on ammonium transformation. Stacked bars indicate percent contribution of nitrification and denitrification to total N₂O emission (denitrification; nitrification) and squares indicate rates of total N₂O emission. Mean ± SE (n = 3). note that x-axis is not in scale.

2. Effect of temperature on nitrification activity

Studying the short-term effects of temperature on N_2O release rates was done by fertilization of the soil with $\text{NH}_4^+\text{-N}$ ($70 \mu\text{g gdw}^{-1}$) followed by incubation for five days (5D treatment) at different temperatures (4, 10, 15, 20, 25 and 37°C) and caused a decrease of ammonium and increase of nitrate concentrations (Fig. 8). The lowest ammonium and highest nitrate concentrations were found at the intermediate temperatures, i.e. $15\text{-}25^\circ\text{C}$. Although these concentrations are also influenced by nitrogen assimilation, denitrification and mineralization of organic nitrogen, they nevertheless indicate that nitrification activity exhibited a maximum at intermediate temperatures.

The same trend was obtained, when ammonium concentrations were measured in EAS soil fertilized with commercial fertilizer at a high (1% w/w; HF treatment) or low (0.3% w/w; LF treatment) concentrations and incubated for long periods (6.5, 16 and 20 weeks) at different temperatures (Fig. 9). Again lowest ammonium concentrations were found at intermediate temperatures.

The conclusion that nitrification was most active at intermediate temperatures was furthermore supported by measurement of potential nitrification activity after 16-20 weeks of incubation (Fig. 10). This activity exhibited a maximum in the intermediate temperature range, especially in the HF treatment.

Rates of N_2O were measured after 5 days of incubation (5D treatment; Fig. 11), in the same way as previously reported by Göttsche and Conrad, (1999) except that the soil had been amended with ammonium. N_2O release was low at 4°C and 10°C (slightly higher at 4°C than at 10°C), increased at $15\text{-}25^\circ\text{C}$ and was highest at 37°C . Contribution of nitrification to N_2O release was in a similar range (35-50%) at all temperatures except at 37°C , where it was only 12% (Fig. 11).

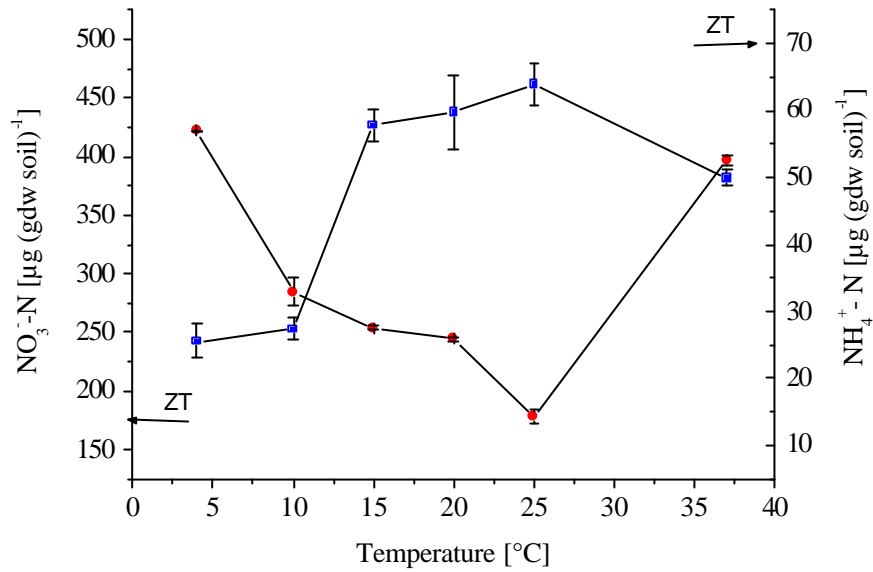


Figure 8: Ammonium and nitrate concentrations after 5 days of incubation at different temperatures. (■ - nitrate concentrations, ● - ammonium concentrations). ZT, soil sample taken before incubation with ammonium. Mean \pm SE; (n = 2).

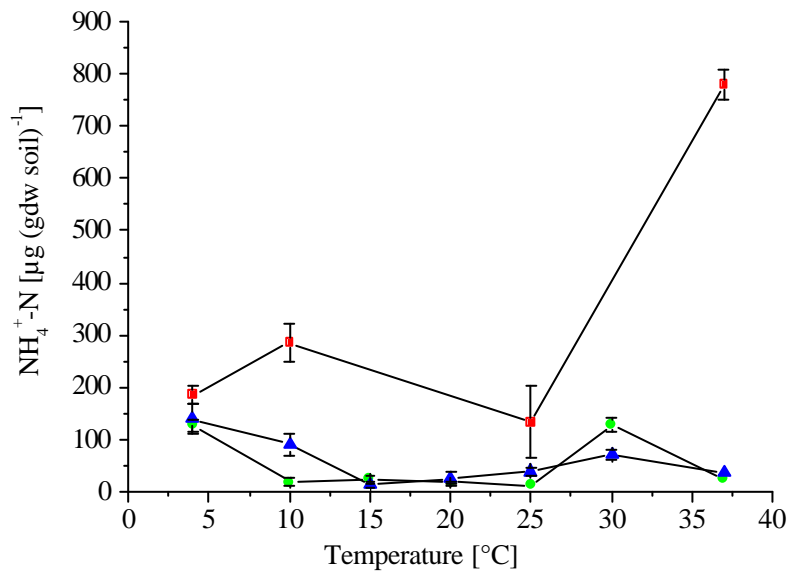


Figure 9: Ammonium concentrations after incubation at different temperatures in high fertilizer (HF) treatment and low fertilizer (LF) treatment. (■ - HF treatments after 6.5 weeks of incubation, ● - LF treatments after 16 weeks of incubation and ▲ - LF treatments after 20 weeks of incubation). Mean \pm SE; (n = 2).

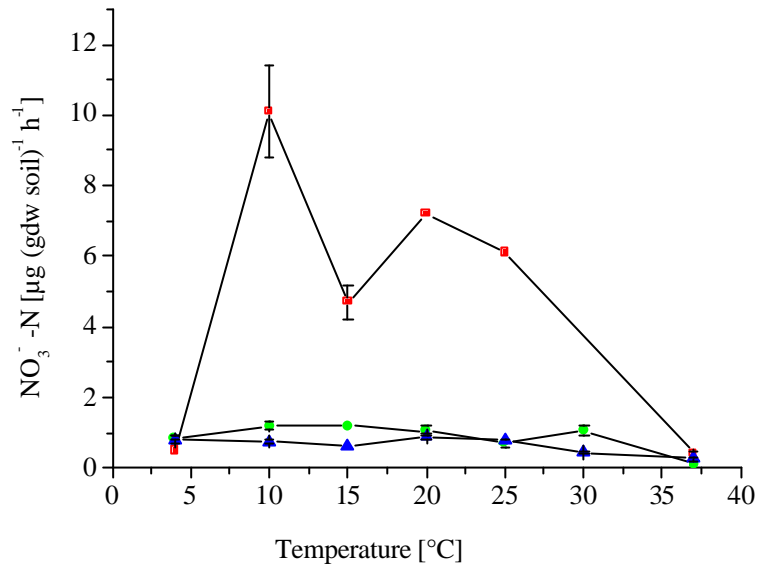


Figure 10: Potential nitrification activity after incubation at different temperatures in high fertilizer (HF) treatment and low fertilizer (LF) treatment. (■ - HF treatments after 20 weeks of incubation, ● - LF treatments after 16 weeks of incubation and ▲ - LF treatments after 20 weeks of incubation). Mean \pm SE (n = 2).

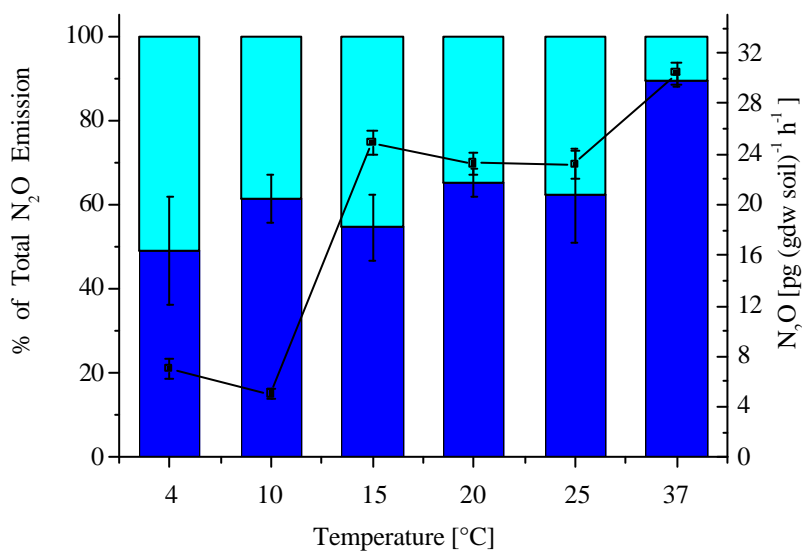


Figure 11: Pattern of N₂O production and percent contribution of nitrification and denitrification to total N₂O emission after 5 days incubation at different temperatures. Stacked bars indicate percent contribution of nitrification and denitrification to total N₂O emission (nitrification; denitrification) and squares indicate rates of total N₂O emission. Mean \pm SE (n=3); note that x-axis is not in scale.

3. Short-term effect of ammonium soil concentration on community structure

The community structure of ammonia oxidizer was analyzed after incubation of EAS soil at low (LA), medium (MA) and high (HA) ammonium concentrations for four weeks at 4°C. The DGGE fingerprints of the amplified *amoA* populations showed no difference among the samples with different ammonium concentrations and the sample (ZT) that was taken from the original soil at the beginning of incubation (Fig. 12). Apparently, the ammonia oxidizer community had not changed significantly during four weeks of incubation at 4°C.

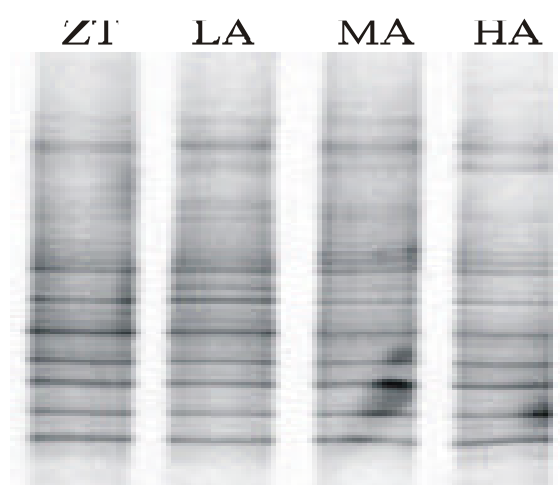
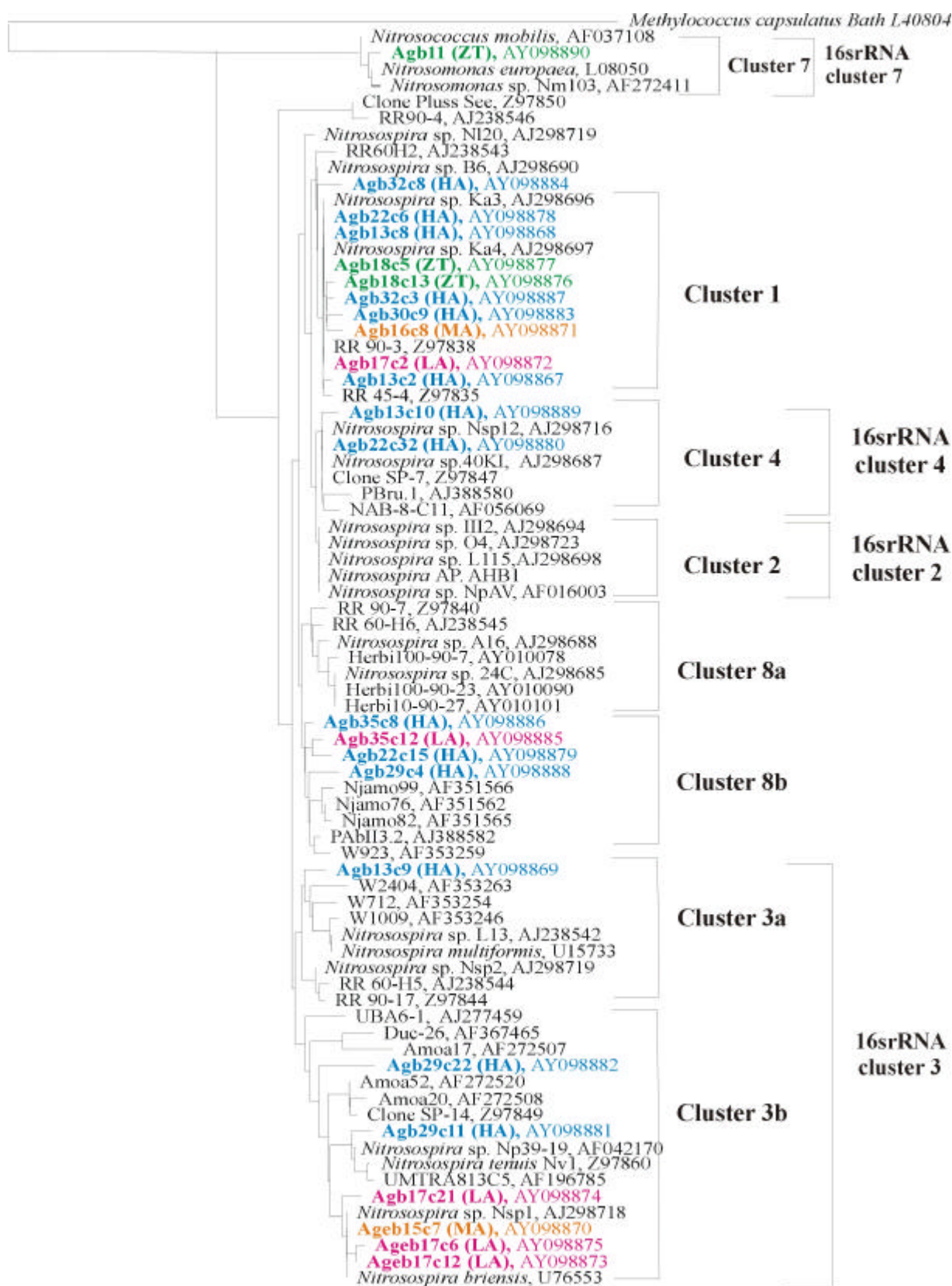


Figure 12: DGGE analysis of *amoA* fragments from soil samples taken before treatment (ZT) and after incubation with low (LA), medium (MA), and high ammonium (HA) concentrations at 4°C for 4 weeks.

Representative bands from the different DGGE lanes were excised, PCR products were cloned and sequenced. The sequences were used to reconstruct phylogenetic trees including other environmental *amoA* clones from the database (Fig. 13). Most of the clones (23 clones) branched with species of the genus *Nitrosospira* and only one clone (Agb11) was affiliated with the genus *Nitrosomonas*, most closely related to *Nitrosomonas* sp. Nm103 (Fig. 13). Clones closely related to *Nitrosospira* cluster with *amoA* from species that belong to cluster 3 and 4 defined by 16S rRNA phylogeny.



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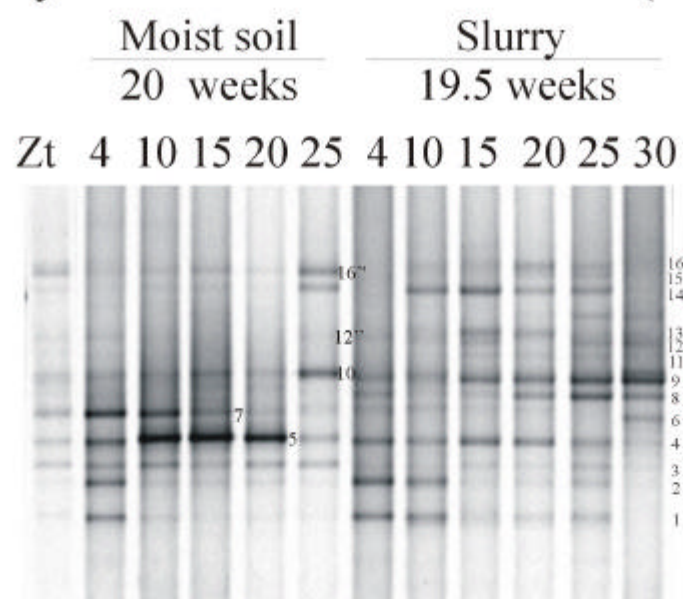
Figure 13: (previous page) Fitch-Margoliash phylogenetic reconstruction (using global rearrangement and randomized input order (3 jumbles) based on partial *AmoA* sequences (150 amino acids). Clones obtained from this experiment are highlighted in bold and in colour. Source of sequences are soil samples taken before treatment (ZT), after incubation with low (LA), medium (MA), and high ammonium (HA) concentrations at 4°C for 4 weeks. Scale bar indicates 10 mutations per 100 sequence positions. Cluster 8a and 8b were defined later as cluster 10 and 9, respectively (see Fig.15).

Additionally, clones clustered with *amoA* from *Nitrosospira* sp. Ka3 and Ka4 (cluster 1) and within an *amoA* cluster of unknown species. The majority of clones occurred within two clusters 1 and 3b. Clones of cluster 1 originated from each of the samples (ZT, LA, MA, HA) while clones of cluster 3b originated from samples LA, MA, and HA. Collectively, these results give no indication for the selection of specific *amoA* clusters by these different incubation conditions. Since no community shift occurred after short incubation as a result of ammonium concentrations, the assumption was that 4 weeks of incubation are too short to detect community shifts of ammonia oxidizers. Therefore, the influence of temperature on the communities of ammonia oxidizers was only evaluated after long period of incubation.

4. Long-term effect of temperature on community structure of ammonia oxidizers

The community structure of ammonia oxidizers in EAS soil was analyzed by DGGE in moist state with LF and HF treatments and in slurries after long-term (6.5, 16 and 20 weeks) incubation at different temperatures. Samples at 37°C did not yield a PCR product in any of the treatments. All the other treatments resulted in diverse *amoA* fingerprint patterns. These variations in the DGGE patterns revealed differences in the composition of the ammonia-oxidizing community between the different treatments (slurry, LF and HF treatments) and a community shift with increasing temperature (Fig. 14). Numbered DGGE bands were excised, purified and sequenced (sometimes after a cloning step). Representative sequences are shown in an *AmoA* tree (Fig. 15). The other clone sequences are only mentioned in the text by giving the clone names in brackets. For designation of the clones see legend of Fig. 15.

a Slurry and moist soil incubation (HF)



b Moist soil incubation (LF)

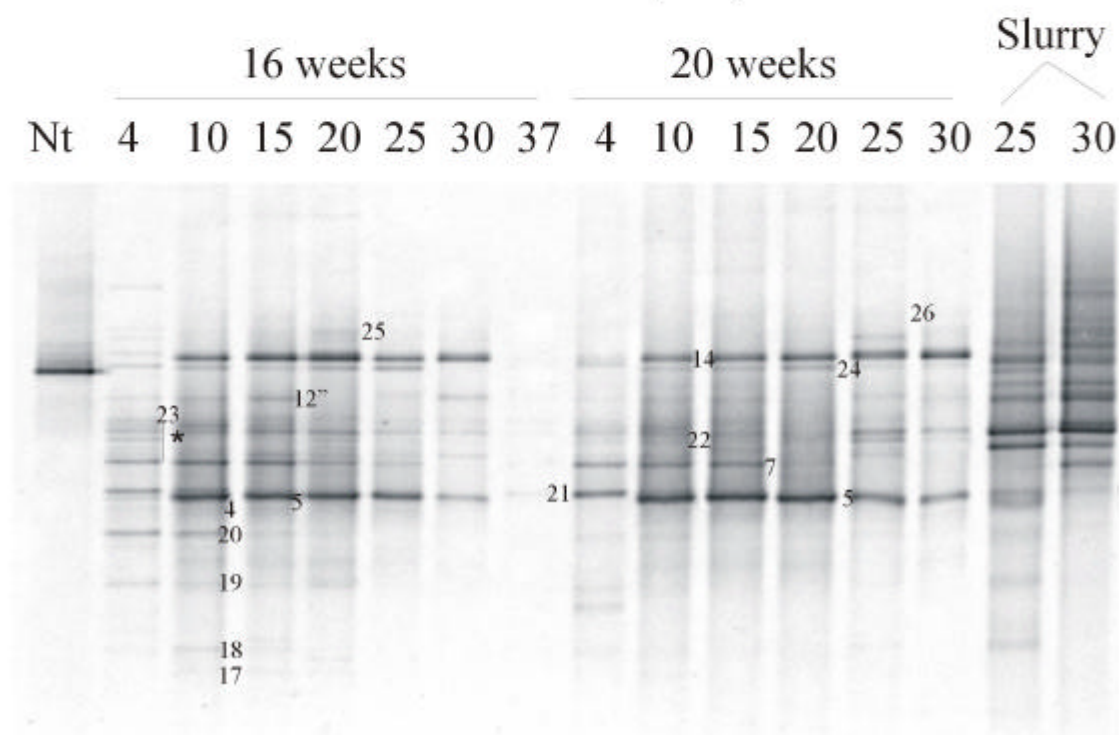


Figure 14 (previous page): DGGE analysis of *amoA* fragments retrieved from EAS soil with (a) high fertilizer (HF) treatment or slurry treatment and (b) low fertilizer (LF) treatment at different temperatures (4 to 37°C) and incubation time. As a comparison two samples of slurry were loaded on the gel with LF samples. The numbering of the bands corresponds to the derived *amoA* sequences highlighted in Fig. 15. Zt represents zero time of the experiment; Nt represents *Nitrosospira tenuis* as reference bacterium.

Most of the bands were only detected in one of the different treatments. Some DGGE bands were detected both in slurry and HF treatment, but not in LF treatment, e.g. bands 1, 2 and 3 (EASHFt10c102, *Nitrosospira* cluster 1; EASHFt4b2 (was not resolved to defined cluster); and EASHFt4b3, *Nitrosospira* cluster 3a) (Fig. 15). Slurry and LF treatment did not share any common band except band 14 (EASLFt15b14, *Nitrosospira* cluster 9), which was the only band detected in all ammonium treatments. Interestingly, *Nitrosospira* cluster 9 included only two DGGE bands derived from HF treatment (band 14 and band 16", EASHFt25b16) (Fig. 15). Bands at the lower part of the DGGE gels showed high intensity at 4°C in HF treatment (bands 1-2, EASHFt10c102 in *Nitrosospira* cluster 1; EASHFt4b2), but were faint in LF treatment (17-19; EASLFt10b17, EASLFt10b18, EASLFt10b19, *Nitrosospira* cluster 1; EASLFt10b20, *Nitrosospira* cluster 3b). These bands were decreasing and disappearing with increase of temperature both in HF and LF treatments, and after 20 weeks of incubation in LF treatment they completely disappeared at all temperatures. On the other hand, some bands like band 7 (EASHFt4b7 or EASLFt10b7, *Nitrosospira* cluster 3b), band 5 (EASLFt20b5 or EASHFt15b5, *Nitrosospira* cluster 3a) and band 12" (EASLFt25b12, *Nitrosospira* cluster 3a) were detected both in HF and LF treatments.

DGGE patterns showed a clear community shift with temperature in each of the ammonium treatments. Bands 5 and 7, which were detected both in LF and HF treatment, decreased in intensity with increase of temperature. Other bands showed the opposite trend with intensity increasing with increase of temperature. For example, bands 10 (EASHFt25c36, *Nitrosospira* cluster 3a) and 16" (EASHFt25b16, *Nitrosospira* cluster 9) in

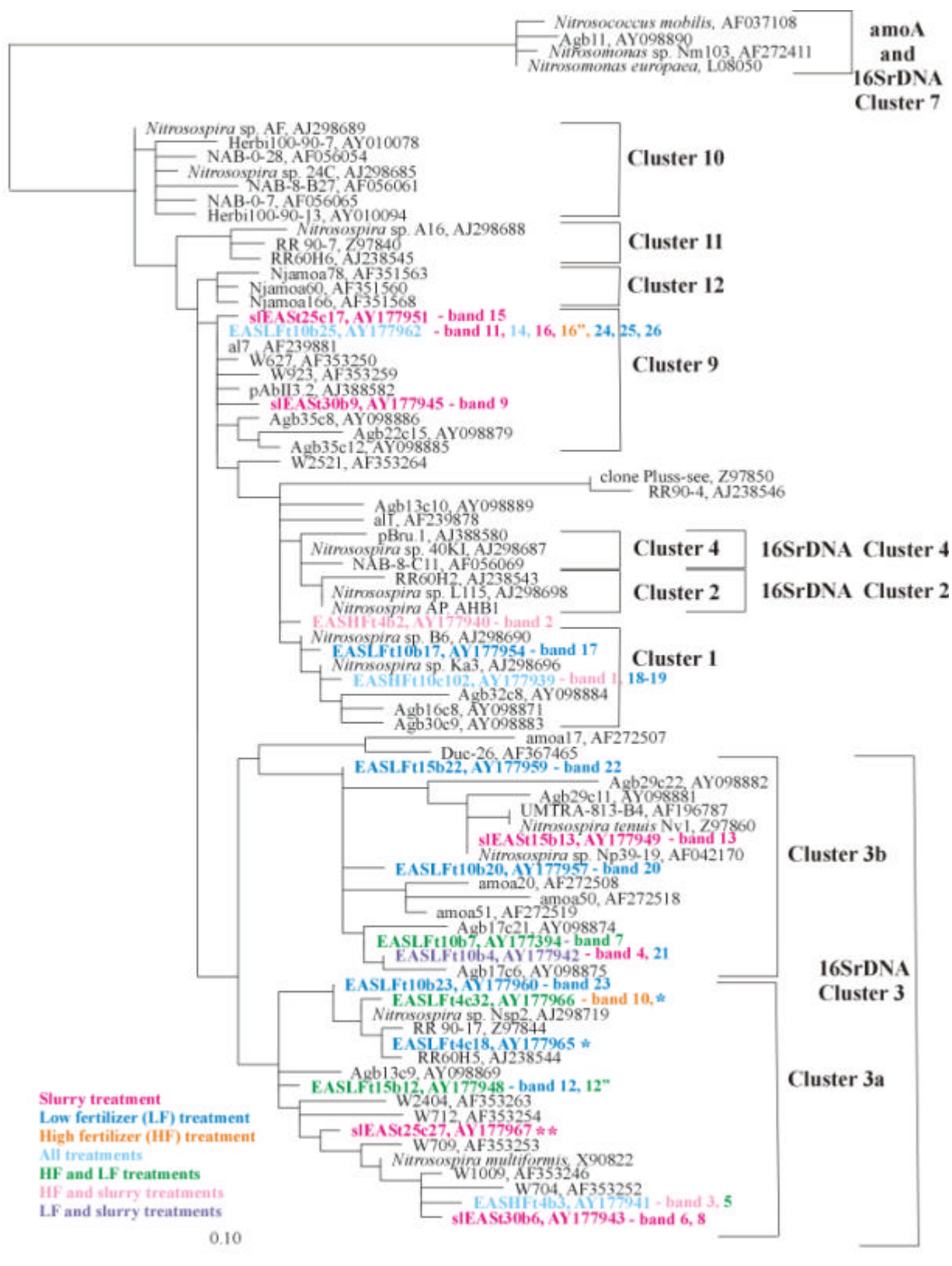


Figure 15 (previous page): Maximum likelihood tree based on partial *AmoA* sequences (150 derived amino acid positions) retrieved from DGGE bands shown in Fig. 14. Source of the *AmoA* sequences are samples taken from slurry (sLEAS), LF treatment (EASLF) and HF treatment (EASHF). Designation of the clones includes the following information: t - temperature of the sample, which the band the clone were was excised or cloned, c – number of clone or b – number of band. Sequences marked with a star were taken from sample that incubated at 4°C after 16 weeks incubation in LF treatment as shown in Fig. 14b. Scale bar indicates 10 changes per 100 nucleotide positions.

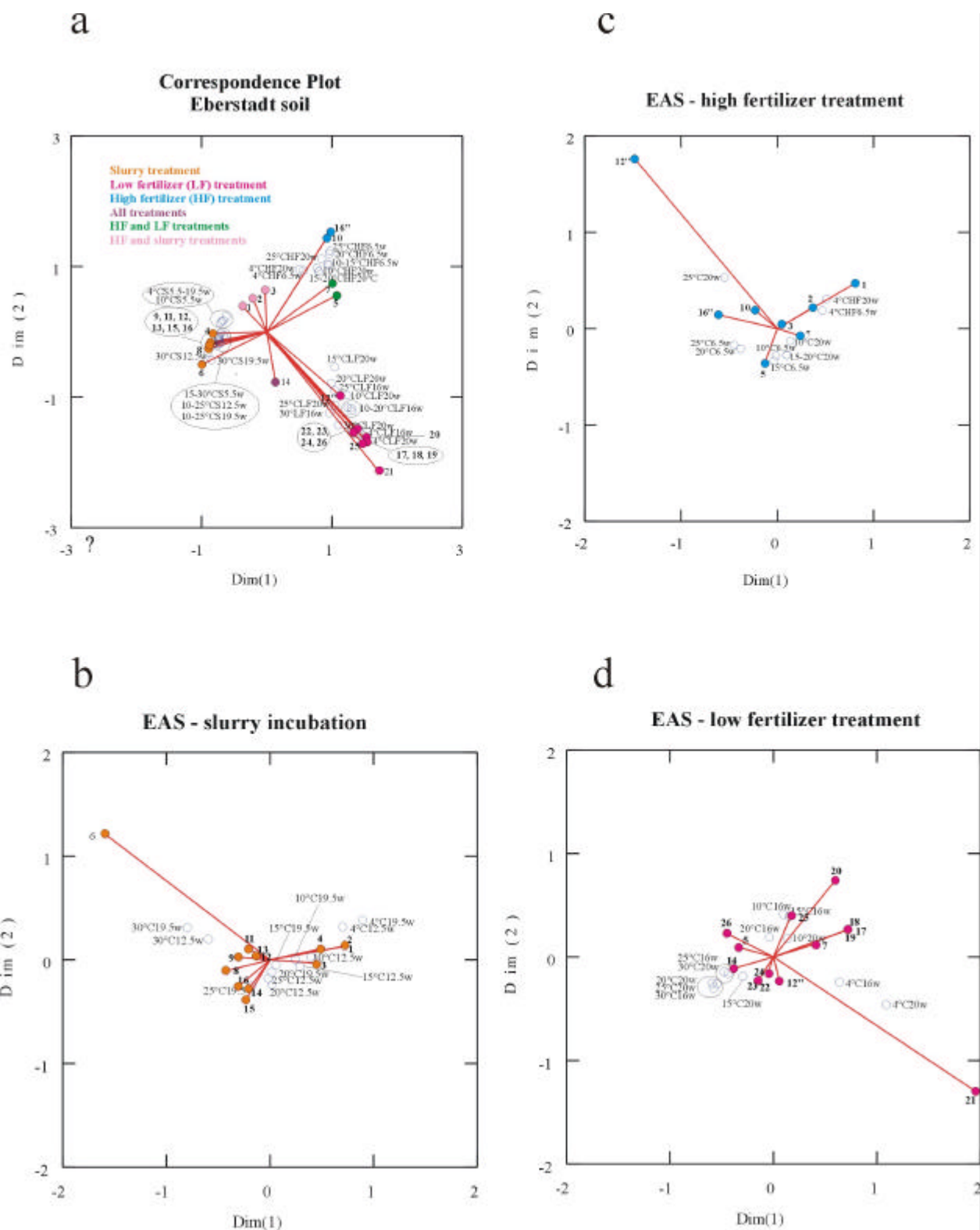
HF treatments had higher intensity at 25°C compared to lower temperatures. Increasing intensity with increase of temperature was observed in slurry incubation for band 8 (sLEASt25b8, *Nitrosospira* cluster 3a) and band 9, (sLEASt30b9, *Nitrosospira* cluster 9).

Correspondence analysis of the DGGE patterns showed that there was significant influence of ammonium treatment on the community structure of ammonia oxidizers in EAS soil (Fig. 16a). The bands, which particularly contributed to the DGGE pattern of the HF treatments, were bands 1-3, 5, 7, 10 and 16'' (grouped in *Nitrosospira* cluster 1, 3a, 3b and 9) (Fig.16a). DGGE bands 1-3 also contributed to the slurry treatment, for which bands 4, 6, 8, 9, 11-13, 15, and 16 (grouped in *Nitrosospira* cluster 3a, 3b and 9) were also important (Fig.16a). In the LF treatment, the DGGE bands 12'', 17-26 (grouped in *Nitrosospira* cluster 1, 3a, 3b and 9) contributed most (Fig.16a). The only correlation between the DGGE banding patterns in the different treatments to specific *amoA* clusters was observed in *Nitrosospira* cluster 1 and 9. Cluster 1 is represented by DGGE bands 1, 17, 18 and 19, while cluster 9 is represented by DGGE bands 9, 11, 14-16, 16'' and 24-26 (Fig. 15). *Nitrosospira* cluster 1 generally contributed in HF treatment, but became less important in LF treatment after long incubation. On the contrary, *Nitrosospira* cluster 9 was generally important at LF treatment and slurry, but became less important in HF treatment after long incubation.

The influence of treatment (HF, LF, slurry) seems to mask the effect of temperature in correspondence analysis (Fig.16a). Only when the different treatments were analyzed

separately, a clear community shift with temperature became visible in the correspondence analysis. In the soil slurry, the DGGE bands that contributed most to the incubations at 25 - 30°C were bands 6, 8, 9 and 11-15 (Fig. 16b). These bands grouped in *Nitrospira* cluster 3a, 3b and 9 (Fig. 15). In contrast, the bands, which contributed most to low temperature incubations, were bands 1-4 (grouped in *Nitrospira* cluster 1, 3a and 3b). HF treatment also showed a community shift with temperature (Fig. 16c). While bands 1-2 showed high contribution in 4°C incubations (band 1 grouped in *Nitrospira* cluster 1), bands 5 and 7 did this in samples incubated at 10 to 20°C (grouped in *Nitrospira* cluster 3a and 3b), and bands 10, 12" and 16' in samples incubated at 25°C (grouped in *Nitrospira* cluster 3a and 9). A trend with temperature was also observed in the LF treatment, where band 21 (EASLFt4b21; *Nitrospira* cluster 3b) had a high contribution at 4°C, bands 7, 17-20 at 4 to 10°C (grouped in *Nitrospira* cluster 1, 3b), bands 12", 22 to 24 (grouped in *Nitrospira* cluster 3a, 3b and 9) at 15°C, and bands 5, 14 and 26 (grouped in *Nitrospira* cluster 3a and 9) at 20 to 30°C (Fig. 16d).

Figure 16 (next page): Correspondence analysis comparing the differences in DGGE banding patterns by the program SYSTAT 9. Open circles represent different samples of EAS soil, which were incubated at different temperatures (4 to 37°C) and/or in different ammonium treatments i.e. (a) all treatments, (b) only slurry treatments, (c) only HF treatments, and (d) only LF treatments. Name of samples composed of °C - temperature, S - slurry, LF - low fertilizer treatment, HF - high fertilizer treatment and w - period of incubation in weeks. Filled circles with a line represent bands with their numbers in bold.



Discussion

Short-term incubation of EAS soil showed a monotonous increase of N_2O release rates with increasing temperatures. This observation is in contrast to previously observed patterns, that N_2O release rates from this soil are relatively high at low temperatures and exhibit a minimum at medium temperatures (Gödde and Conrad, 1999). The discrepancy may be resolved by the fact that initial ammonium concentrations were much higher in the present than in the earlier study. Since ammonium was consumed more rapidly at medium than at low temperatures, ammonium may have become limiting for N_2O release at medium temperatures in the earlier but not in the present study. The same pattern of low ammonium concentrations at medium temperatures compared to low and high temperatures was observed in the long-term incubation experiments (HF and LF treatments) and was consistent with the potential nitrification activity that showed a maximum at intermediate temperatures. The pattern of potential nitrification activity also reflected that of the contribution of nitrification to N_2O release. Slight discrepancies may be due to the fact that potential nitrification was measured after much longer incubation than N_2O release, so that community shifts of ammonia oxidizers may have occurred (see below). Another difference to the previous study of Gödde and Conrad, (1999) was that denitrification instead nitrification was the major source of N_2O , accounting for >55% of total N_2O release. This result was probably also caused by the relatively high soil ammonium content which allowed the accumulation of nitrate and thus enabled nitrate reduction activity. In summary, our short-term incubation experiments indicate that temperature-dependent potential nitrification activities may result in limitation of ammonium, which in turn limits rates of N_2O release. Hence, a shift in the community of ammonia oxidizers is not required to explain the minimum of N_2O release at medium temperatures found by Gödde and Conrad, (1999). Indeed, analysis of the community structure of ammonia oxidizers after short incubation at different ammonium concentrations did not show any community shift after 4 weeks of incubation, although nitrification activity increased upon addition of ammonium.

Similar to temperature, increase of ammonium concentrations resulted in increase of N_2O release rates by EAS soil after short-term incubation. With medium and high ammonium concentration we also observed a shift towards a higher contribution of nitrification to N_2O production compared to soil after addition of low concentration of ammonium. These results are in agreement with other studies (Schuster and Conrad, 1992; Müller et al., 1998; Skiba and Smith, 2000). Studying the soil ammonia oxidizer population represented by DGGE patterns of the *amoA* gene showed no significant change during four weeks of incubation at 4°C, even when the soil was amended with high ammonium concentrations. Therefore, the response of the activity was probably not the result of a major change in the ammonia-oxidizing population but of a physiological shift. Mendum et al. (1999) arrived at a similar conclusion studying ammonia-oxidizing populations in fertilized and unfertilized soil over a period of six weeks. This conclusion was further supported by analysis of the phylogenetic structure of *amoA* sequences retrieved from the soil ammonia-oxidizing community which did not reveal any clustering of genes with respect to ammonium treatment. In contrast, our analysis revealed an amazingly high diversity of *amoA* sequences from the different ammonium treatments, affiliated with five *Nitrosospira* clusters and one *Nitrosomonas* cluster. No clones were affiliated with *Nitrosospira* AP. AHB1 from cluster 2, which was isolated from an acidic soil (Stephen et al., 1998; Kowalchuk et al., 2000b). It should also be noted that all *amoA* sequences had a similarity of >85% (amino acid sequence level) to that of known ammonia oxidizer species. Hence, all the diversity was displayed within a relatively narrow range of species. Generally, diversity of ammonia oxidizers in tilled agricultural soils is expected to be much lower than in native soils (Bruns et al., 1999). However, EAS soil had been treated with composted organic material briefly before sampling, which may have led to an input of organisms to the native ammonia-oxidizer populations. Obviously, ammonia oxidizers grow so slowly that changes in their community cannot be detected within 4 to 6 weeks of incubation. Our long-term incubation experiments, on the other hand, demonstrated

that community shifts of ammonia oxidizers did occur after 16 weeks of incubation, and that fertilization and temperature affected the pattern of community shift.

Long-term incubation of EAS soil at different temperatures and in different experimental set-ups (HF, LF, slurry) showed clear shifts of the *amoA* fingerprint patterns. Correspondence analysis indicated a strong influence of the incubation set-up on the community structure of ammonia oxidizers. Most of these community shifts seemed to occur within the different *Nitrosospira* clusters, since all clusters contained sequences of DGGE bands retrieved from both HF and LF treatments. Nevertheless, there seemed to be also community shifts between *Nitrosospira* clusters. For example, DGGE bands related to *Nitrosospira* cluster 1 were more intense in the HF treatment, while they completely disappeared after long period of incubation in the LF treatment. We suggest that ammonia oxidizers from *Nitrosospira* cluster 1 are more sensitive to low ammonium concentrations compared to those from other clusters and probably do not grow below a certain threshold of ammonium concentrations. A similar dependency on ammonium concentrations was recently found during enrichment of particular *Nitrosomonas* species (Bollmann and Laanbroek, 2001; Bollmann et al., 2002). DGGE bands clustering in *Nitrosospira* cluster 3 were detected both in HF and LF treatments indicating that *Nitrosospira* cluster 3 is not necessarily dominant at high ammonium concentrations as suggested in previous studies (Bruns et al., 1999; Kowalchuk et al., 2000a; 2000b). Interestingly, *Nitrosospira* cluster 9 included only two bands derived from HF treatment (band 14, EASLFt15b14; and band 16'', EASHFt25b16). These bands were detected after 20 weeks of incubation at 25°C, while ammonium concentration was the lowest compared to other temperatures of HF treatment (ca. 130 µg ammonium-N gdw soil⁻¹). *Nitrosospira* cluster 9 was also detected in irrigated agriculture soil, which was treated with low ammonium concentrations (Oved et al., 2001).

The effect of temperature on ammonia oxidizer community structure was clearly seen when focussing on individual bands in the DGGE patterns. Some bands were decreasing with temperature (like bands 5 and 7), while others were increasing with temperature (like bands 8, 9, 10 and 16"). In correspondence analysis, however, the effect of fertilizer treatment masked that of temperature. On the other hand, correspondence analysis separately for each treatment revealed the effect of temperature on community structure. In addition, slurry incubation, in which pH, ammonium and water activity were under control, showed a clear community shift that must be due to temperature effect. However, most of the temperature effect apparently caused population shifts within the different *Nitrosospira* clusters. Only ammonia oxidizers belonging to *Nitrosospira* cluster 1 were more prevalent at low temperature (4 to 10°C) in HF treatment and slurry, while they completely disappeared at high temperature (30°C).

In conclusion, results show that N₂O emission rates from soil are positively correlated to soil ammonium concentrations and temperature. Community shifts after short incubation were negligible for ammonia oxidizers. Thus, adaptation occurs rather on the level of physiological status than by community shifts. However, after a long period of incubation (16 weeks) EAS soil showed shifts in the ammonia oxidizer community. These community shifts were affected by different fertilizer treatments and incubation temperature, but were mostly within the different phylogenetic clusters of *Nitrosospira*, thus offering population shifts between closely related ammonia oxidizers. Only *amoA* clusters 1 and 9 might be affected in total, as DGGE bands belonging to these clusters were more prevalent at low temperature and high ammonium concentrations or at low ammonium concentrations, respectively. For cluster 9 there are no pure cultures available yet, and for cluster 1 only recently pure cultures were isolated (Aakra et al., 1999b), so that the phenotype of these ammonia oxidizers is unclear. Further studies are necessary to understand community changes of ammonia oxidizers in soils and further studying of temperature effect on ammonia oxidizers is described in chapter 3 of this thesis.

Chapter 3:

**Long-term effect of temperature on community structure
of ammonia oxidizers in different meadow soils**

Results

1. Ammonium measurements

Ammonium concentrations in all soils were at least 130 times higher than those in field soil samples (Table 2), and were increasing with temperature after incubation (Fig. 17). The concentrations after 16 and 20 weeks incubation of KMS soil were high and ranged between 210 ± 65 to $1570 \pm 90 \mu\text{g NH}_4^+\text{-N gdw soil}^{-1}$. In OMS soil the concentrations were significantly lower in the LF treatment ($< 215 \mu\text{g NH}_4^+\text{-N gdw soil}^{-1}$) than in HF treatment ($< 550 \mu\text{g NH}_4^+\text{-N gdw soil}^{-1}$). Since there was no overlap of the range between HF and LF treatments, soil condition could be defined also as high and low ammonium concentrations, respectively. Another important factor that was found for KMS soil was pH, which decreased during incubation at all temperatures above 4°C from pH 7.9 in the field to values of 6.2-6.6.

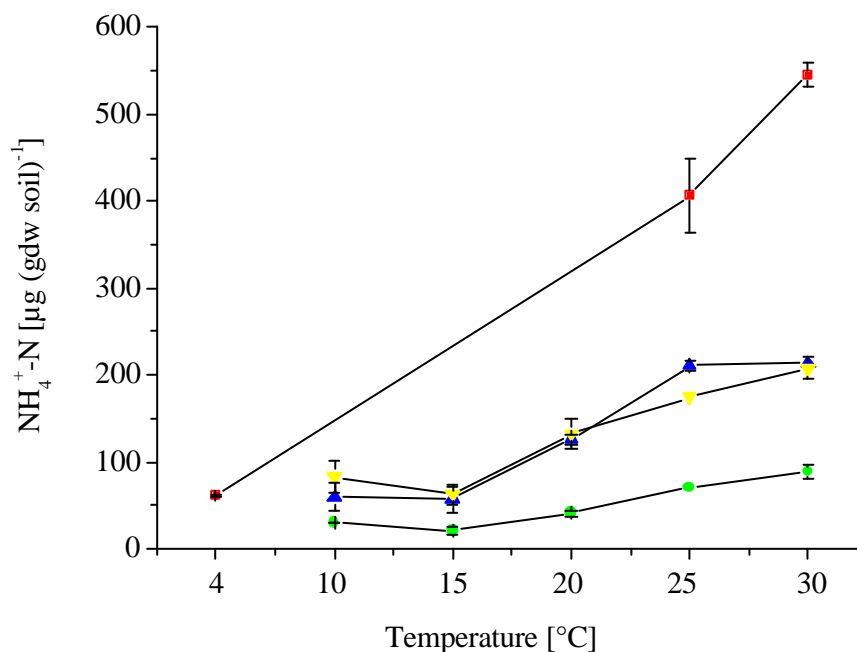


Figure 17: Ammonium concentrations after incubation at different temperatures in high fertilizer (HF) treatment and low fertilizer (LF) treatment of OMS soil (Oppenrod, Germany). (■ - HF treatments after 6.5 weeks of incubation, ● - LF treatments after 8 weeks of incubation, ▲ - LF treatments after 16 weeks of incubation and ▼ - LF treatments after 20 weeks of incubation). Mean \pm SE (n=2).

2. Potential nitrification activity

Potential nitrification activity of all soils was measured after incubation for 20 weeks. KMS and GMS soils were rather active (optimum of 8.4 ± 1.15 and $6.6 \pm 0.4 \mu\text{g NO}_3^- \text{-N gdw soil}^{-1} \text{ h}^{-1}$, respectively) (Fig. 18). Lowest activity of all soils was measured at 4°C (between 0.35 ± 0.2 and $1.15 \pm 0.07 \mu\text{g NO}_3^- \text{-N gdw soil}^{-1} \text{ h}^{-1}$). The potential activities of OMS soil both in HF and LF treatments were very low ($< 1.1 \pm 0.2 \mu\text{g NO}_3^- \text{-N gdw soil}^{-1} \text{ h}^{-1}$), although values of HF treatment of this soil were at least two times higher compared to those of LF treatment.

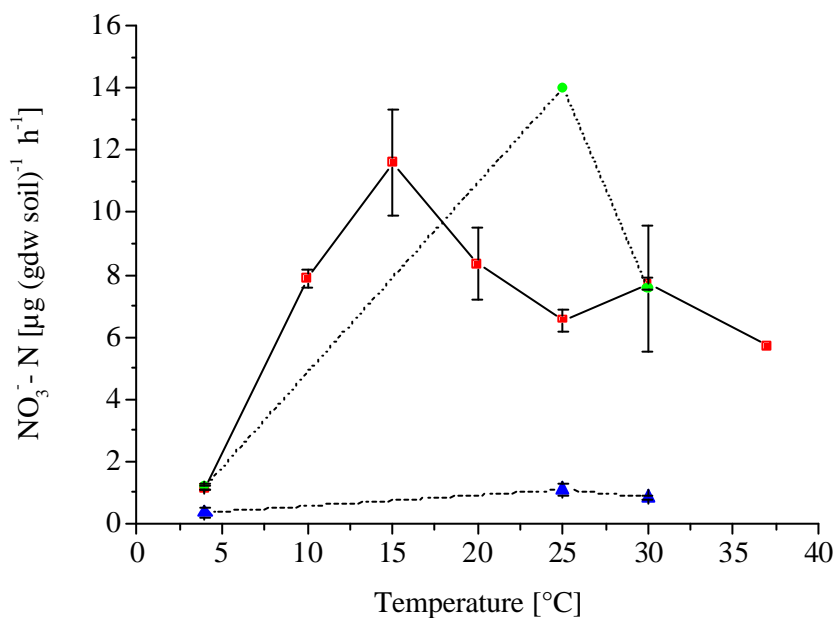


Figure 18: Potential nitrification activity after 20 weeks of incubation at different temperatures.

■ - KMS soil, ● - GMS soil, ▲ - OMS soil high fertilizer (HF) treatment. Mean \pm SE (n=2).

3. Molecular analysis of environmental samples

Samples from the three meadow soils were taken for molecular analysis after field sampling. DNA was extracted, PCR products of *amo A* gene from all soils were cloned, and clones were compared by DGGE with their environmental sample. Interestingly, majority of sequences from KMS soil grouped together in *Nitrosospira* cluster 3a (Fig. 19).

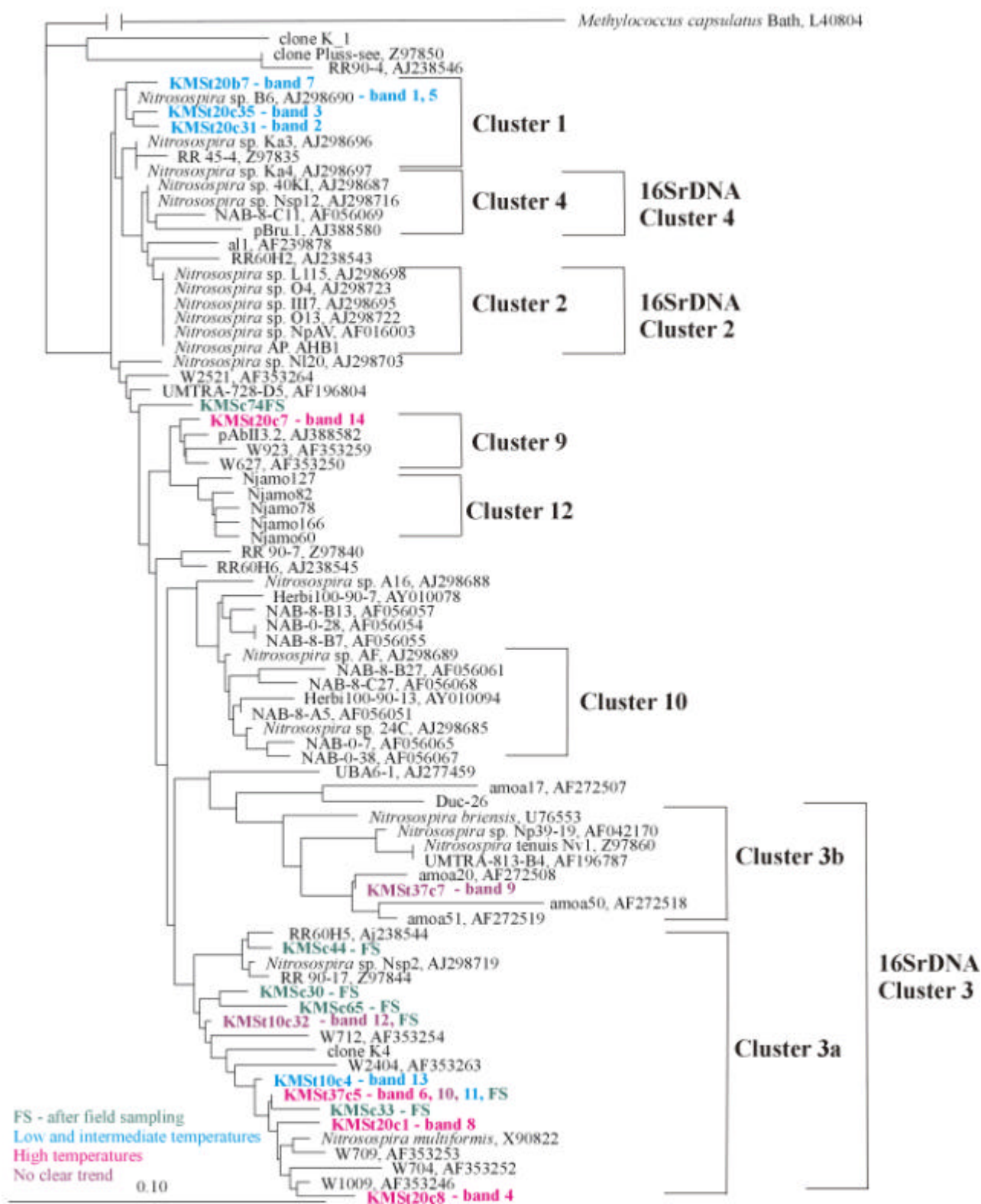


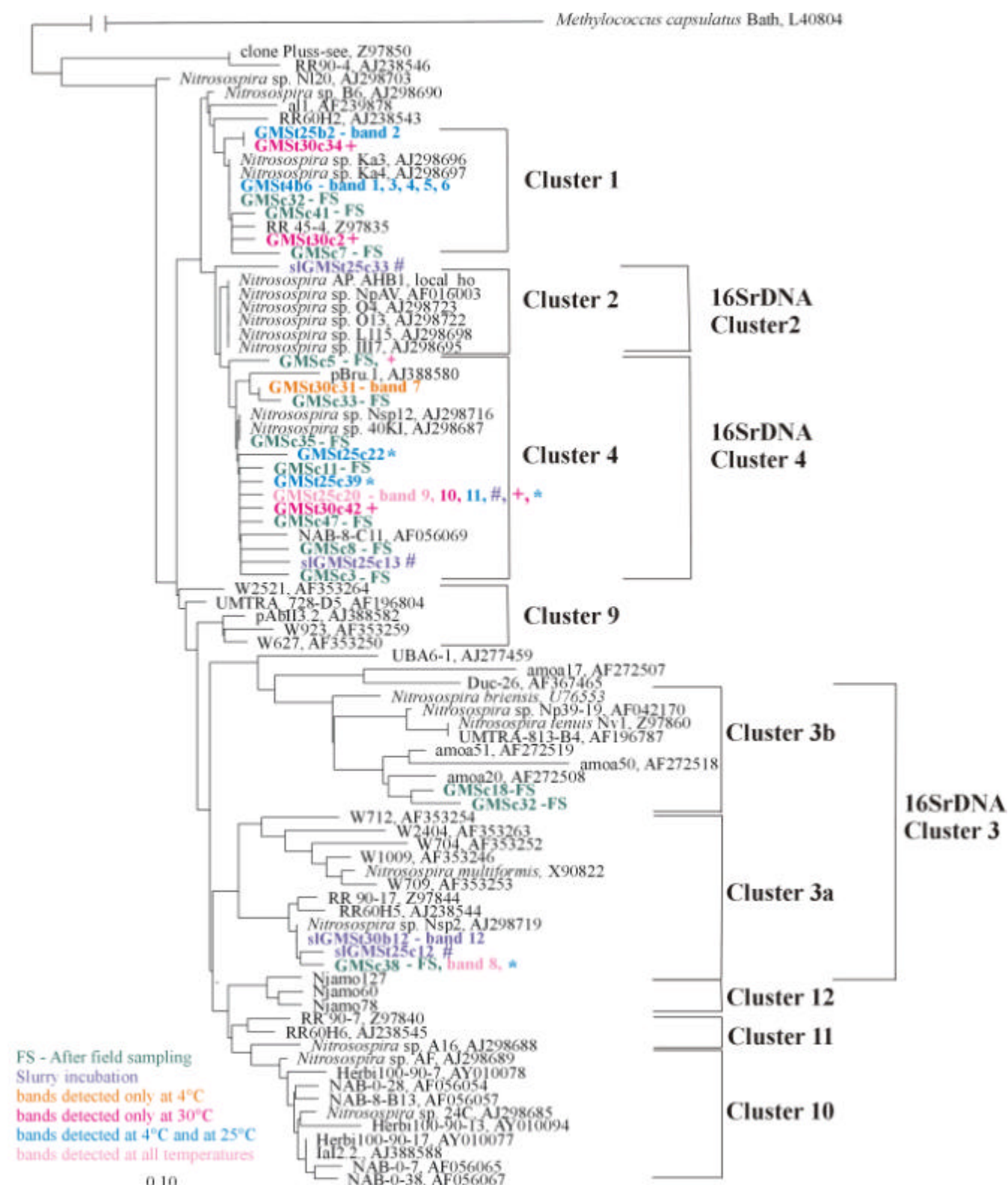
Figure 19: (previous page) Fitch-Margoliash phylogenetic reconstruction (using global rearrangement and randomized input order (3 jumbles) based on partial *AmoA* sequences (150 amino acids) retrieved from field sample and DGGE bands shown in Fig. 22. Clones and bands obtained from this experiment are highlighted in bold and in colour. Source of the *AmoA* sequences are samples taken from the field KMS (FS) or after incubation in moist state (KMS). Designation of the clones includes the following information: t - temperature of the sample, which the band or the clone were excised or cloned, c - number of clone or b - number of band. Scale bar indicates 10 changes per 100 nucleotide positions.

Sequences from GMS and OMS soils showed a higher diversity than those from KMS soil. However, the majority of sequences retrieved from GMS soil grouped in *Nitrosospira* cluster 1 and 4. Four additional sequences grouped in *Nitrosospira* cluster 3a and 3b (Fig. 20). Sequences retrieved from OMS soil grouped in *Nitrosospira* cluster 1 (three of them above 99% similarity), *Nitrosospira* cluster 3b, and were closely related to an environmental sequence without any representative pure culture (all-like sequences) (Fig. 21).

4. Molecular analysis of moist soil and slurry incubation

Samples from all soils were taken for molecular analysis after incubation at different temperature. DNA was extracted and the PCR products of the *amoA* gene in samples incubated at different treatments were compared by DGGE. Moist soil samples of each soil were compared with slurry samples of the same soil.

Figure 20: (next page) Fitch-Margoliash phylogenetic reconstruction (using global rearrangement and randomized input order (3 jumbles) based on partial *AmoA* sequences (150 amino acids) retrieved from field sampling and DGGE bands shown in Fig. 24. Clones and bands obtained from this experiment are highlighted in bold and in colour. Source of the *AmoA* sequences are samples taken from the field GMS (FS) or after incubation in moist soil (GMS), or after incubation in slurry (slGMS). Symbols represents: + upper faint bands at 30°C of moist soil, * upper faint bands at 25°C of moist soil, # upper faint bands at 25°C of slurry. Designation of the clones includes the following information: t - temperature of the sample, which the band and the clone were excised or cloned, c - number of clone or b - number of band. Scale bar indicates 10 changes per 100 nucleotide positions.



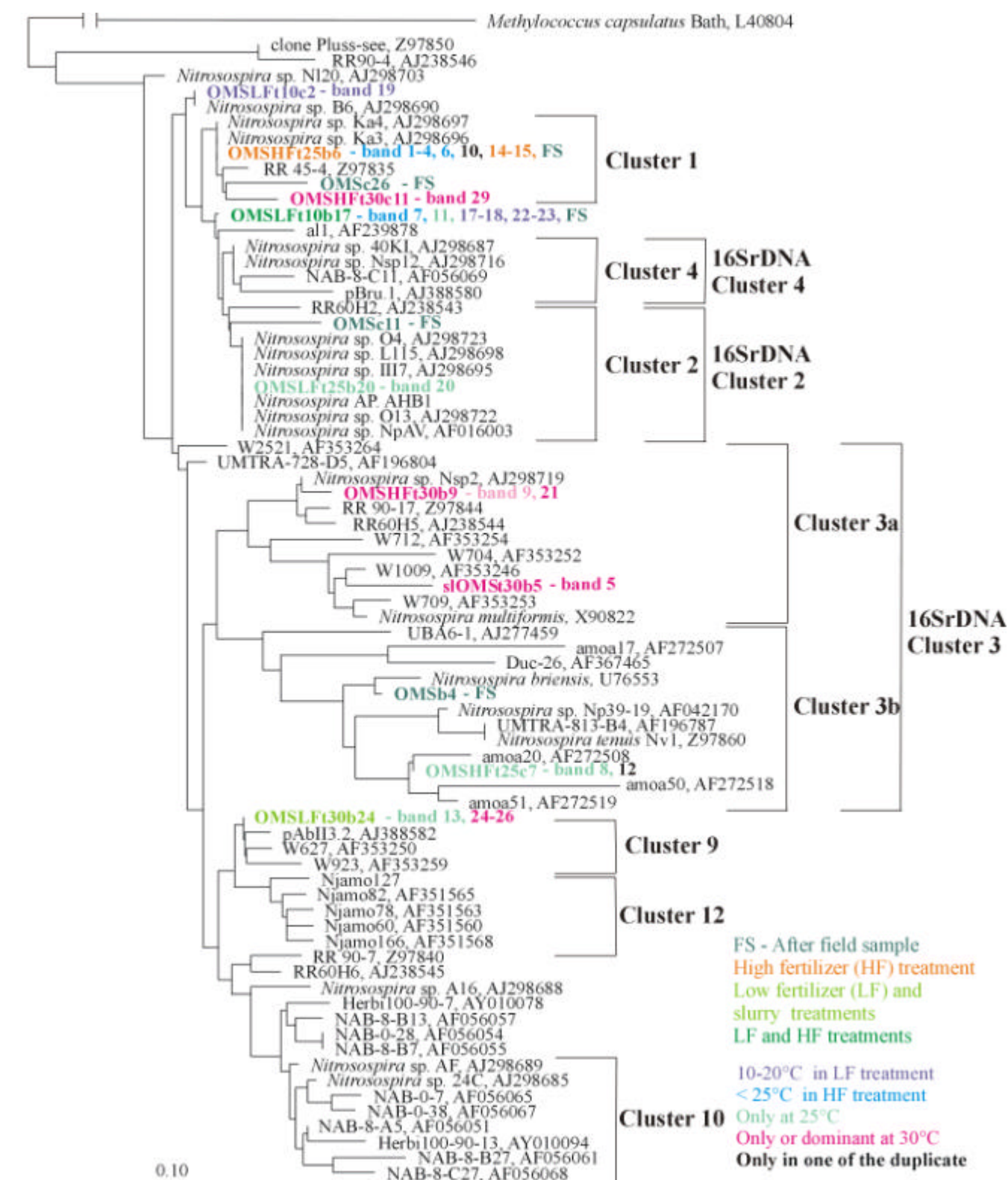


Figure 21: (previous page) Fitch-Margoliash phylogenetic reconstruction (using global rearrangement and randomized input order (3 jumbles) based on partial *AmoA* sequences (150 amino acids) retrieved from field sample and DGGE bands shown in Fig. 25. Clones and bands obtained from this experiment are highlighted in bold and in colour. Source of the *AmoA* sequences are samples taken from the field OMS (FS) or after incubation in moist soil (OMS), or after incubation in slurry (slOMS). Designation of the clones includes the following information: t - temperature of the sample, which the band the clone were was excised or cloned, c - number of clone or b - number of band. Scale bar indicates 10 changes per 100 nucleotide positions.

4.1. KMS soil

The community structure of the ammonia oxidizers in KMS soil after incubation in moist state was analyzed by DGGE of the *amoA* gene, and showed a clear shift with temperature (4-37°C) (Fig. 22). While the intensity of some DGGE bands was increasing with increase of temperature, the intensity of other bands was decreasing. However, all these sequences were grouped in *Nitrosospira* cluster 3a (Fig. 19), similar to the sequences of this soil after field sampling. For example, bands 4 and 8 (KMSt20c8/KMSt30b4 and KMSt20c1, respectively) were increasing with the increase of temperature. Band 8 was the only detectable band at 37°C after 20 weeks of incubation (DGGE is not shown), although after 16 weeks of incubation band 6 (KMSt37c18) was detected at 37°C as well. On the contrary, band 11 and 13 (KMSt37c5 and KMSt10c4, respectively) were decreasing with increase of temperature and completely disappeared at 30°C or at 20°C, respectively. The soil slurries showed similar trends but exhibited a lower diversity compared to moist soil incubation experiments. For example, band 4 and 8 were detected only at 30°C or at 20 - 30°C, respectively, while band 11 was very intense at 4°C, but was not detected at the other temperatures.

Higher diversity was observed at the intermediate temperatures (15 – 20°C) in moist soil samples of KMS, while DGGE bands 1-3 showed up in these samples (KMSt20c6, 99% similarity to *Nitrosospira* sp. B6; KMSt20c31 and KMSt20c35, respectively). However, bands 1 and 3 were also detected at 10°C or at 30°C, respectively, after 20 weeks of incubation, but were much fainter. Two more bands, which were detected at low and at the

intermediate temperatures, were band 5 (KMSt10c1, 99% similar to *Nitrosospira* sp. B6, at 15°C) and band 7 (KMSt20b7, 4 - 20°C). Sequences of all these bands grouped in *Nitrosospira* cluster 1. Band 14 (KMSt20c7), which could be detected only at 25-30°C, was the only band whose representative sequence grouped in *Nitrosospira* cluster 9. Correspondence analysis of the DGGE patterns showed that there was a significant influence of temperature treatment on the community structure of ammonia oxidizers in KMS soil (Fig. 23).

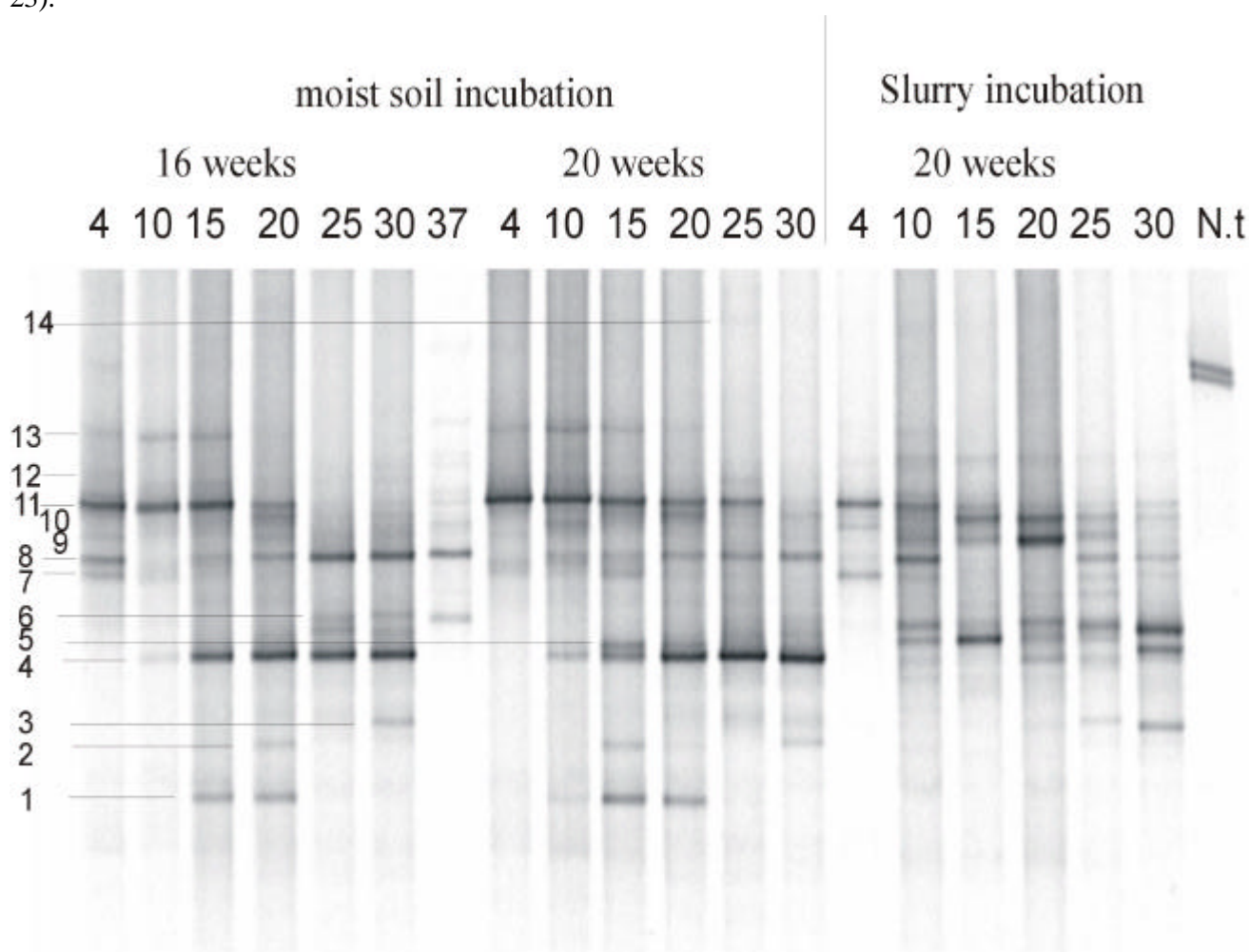


Figure 22: DGGE analysis of *amoA* fragments retrieved from KMS soil after 16 or 20 weeks of incubation at different temperatures. Samples are representing (as indicate) a moist soil or slurry after 16 or 20 weeks of incubation at different temperature (4-37°C). The numbering of the bands corresponds to the derived *AmoA* sequences highlighted in Fig. 19. Nt represents *Nitrosospira tenuis* as reference bacterium.

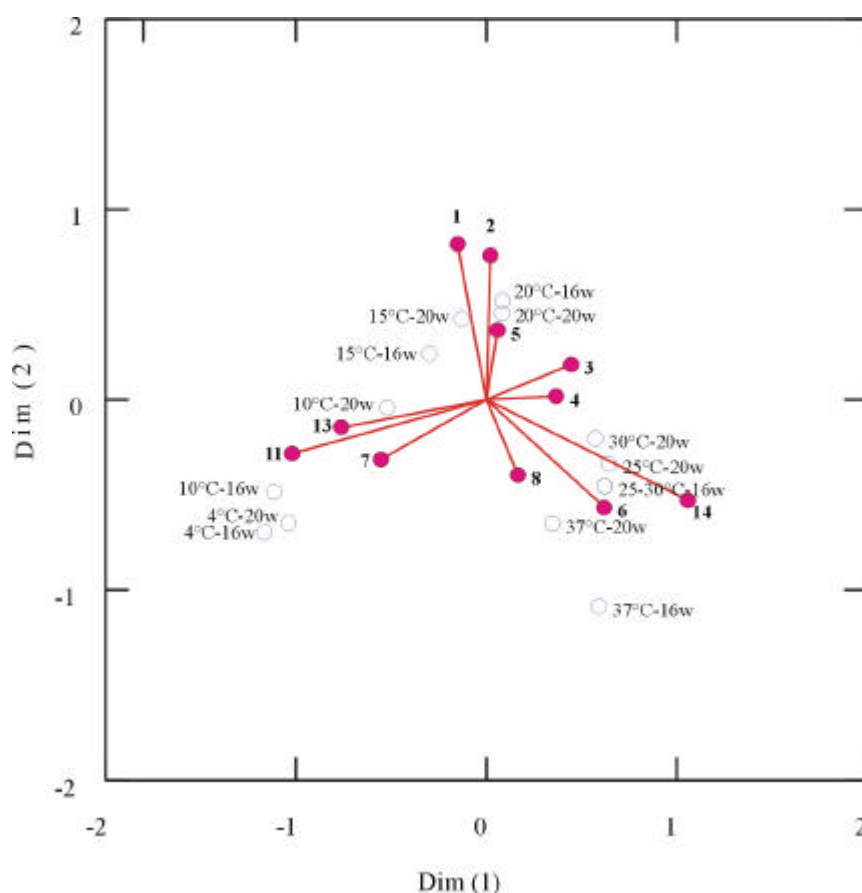


Figure 23: Correspondence analysis comparing the differences in DGGE banding patterns by the program SYSTAT 9. Open circles represent different samples of KMS soil, which were incubated at different temperatures (4 - 37°C) in moist soil state. Name of samples composed of °C - temperature, w - period of incubation in weeks. Filled circles with a line represent bands with their numbers in bold.

4.2. GMS soil

DGGE pattern of the *amoA* gene after 6.5 weeks incubation in moist soil at different temperatures showed mainly differences in the band intensity (Fig. 24). However, after 20 weeks incubation marked differences developed. The pattern at 30°C was significantly different from that at lower temperatures. The dominant band at 30°C (band 8, GMSt30c4, in *Nitrosospira* cluster 3a) was also detected at lower temperatures but was much fainter. At 30°C no bands could be detected at the lower part of the gel. In the upper part of the gel the

pattern at 30°C was different from that at 4°C and 25°C. The majority of bands, which were detected only at 4°C and 25°C, but not at 30°C, grouped in *Nitrosospira* cluster 1. One of these bands (No. 6) was excised from samples at 4 and 25°C (GMSt4b6 and GMSt25b6, respectively). The two sequences showed only 2 bp differences and no difference in the amino acid sequence. Most of the faint bands in the upper part of the gel at 30°C (marked as +, Fig. 24: GMSt30c2 and GMSt30c34 in *Nitrosospira* cluster 1; GMSt30c42 and GMSt30c11 (99% similarity to each other), GMSt30c37, GMSt30c5 and GMSt30c5c21; The last three sequences are not shown in the tree since the first was 99% similar to GMSc5, and the last two to GMSt25c20, in *Nitrosospira* cluster 4) (Fig. 20) were not reproducible in different DGGE gels, probably being close to the detection limit of PCR. The differences between 4°C to 25°C were mainly by intensity of bands, while bands in the upper part of the gel (marked as *, Fig. 24, in *Nitrosospira* cluster 4) were more intense at 25°C than at 4°C. The only difference between 4°C and 25°C was band 7 (GMSt30c31, in *Nitrosospira* cluster 4) detected only at 4°C (Fig. 20).

Similar to moist soil incubation, DGGE patterns of slurry incubation showed mainly differences between 30°C and the lower temperature treatments (4 and 25°C) (Fig. 24). After 12.5 weeks of incubation at 30°C only one band was detectable (band 8, slGMSt30b8). The faint bands in the upper part of the gel at 25°C (signed as #, Fig. 24) were sequenced, and grouped in *Nitrosospira* cluster 3a and 4. Correspondence analysis of this soil supported the clear trend of individual bands with incubation temperature, but did not add additional information to the observation shown by DGGE banding pattern (data are not shown).

4.3. OMS soil

Similar to GMS soil, DGGE pattern of *amoA* gene after incubation of OMS soil in HF moist state treatments showed differences only after 20 weeks incubation. The main differences were observed in incubations at 30°C, which were characterized by low diversity (only two bands). One of the two bands was also detected at 4 and 25°C (band 9,

OMSHFt30b9, in *Nitrosospira* cluster 3a), but was less intense than at 30°C, while the other band was not detected at any other temperature (band 4, OMSHFt30c3, in *Nitrosospira* cluster 1). On the contrary, no marked differences were observed in DGGE patterns at 4°C and 25°C after 20 weeks of incubation (Fig. 25a), except the intensity of bands, and band 8 (OMSHFt25c7, in *Nitrosospira* cluster 3b), which could be detected only at 25°C (Fig. 25a; Fig. 21). The low intensity of bands at 4°C compared to bands at 25°C was in agreement with the higher potential nitrification activity of the samples at 25°C compared to 4°C.

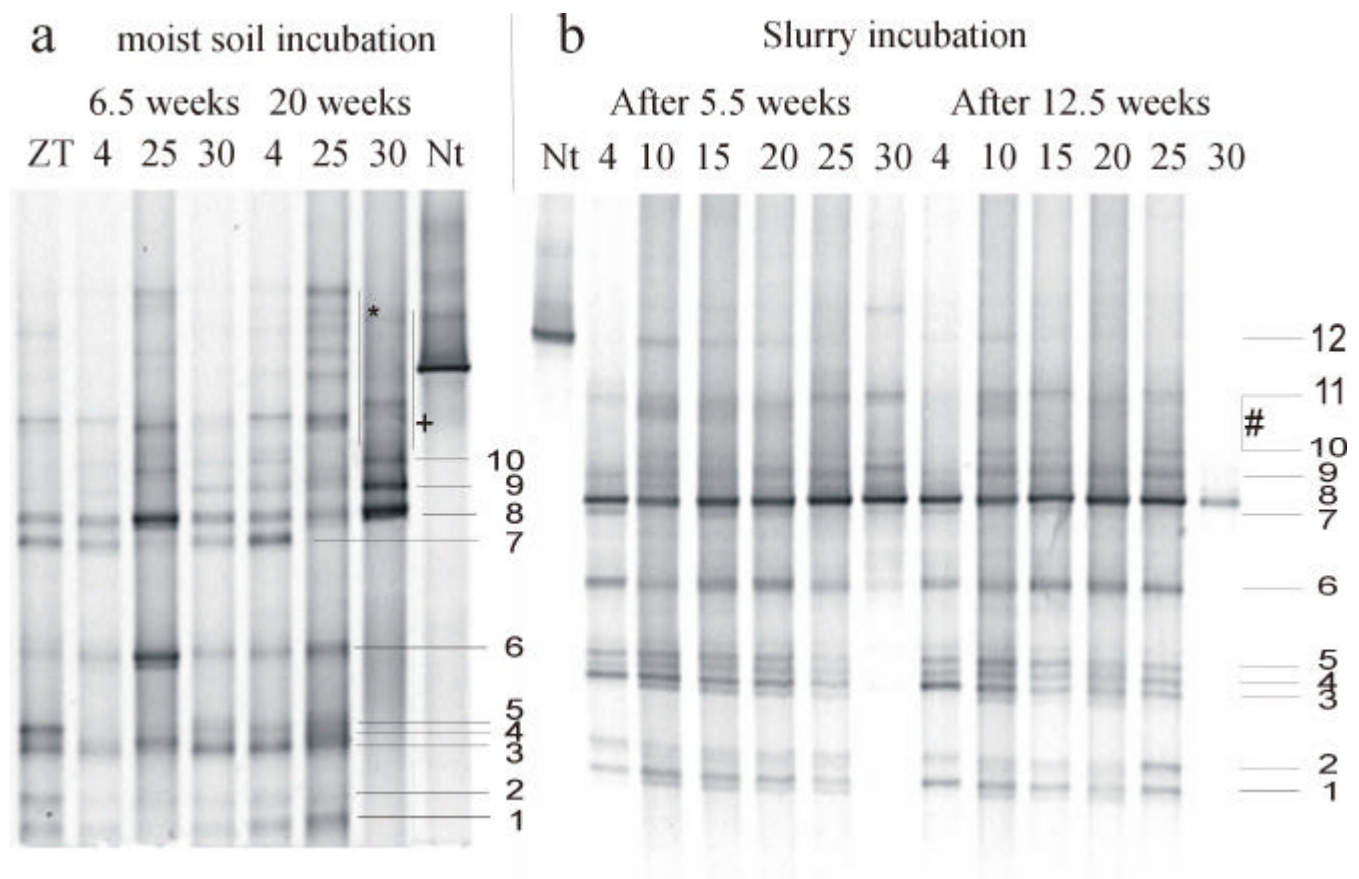


Figure 24: DGGE analysis of *amoA* fragments retrieved from GMS soil (a) moist soil incubation (b) slurry incubation at different temperatures (4 to 30°C) and incubation time. The numbering of the bands corresponds to the derived *AmoA* sequences highlighted in Fig. 20. Zt represents zero time of the experiment; Nt represents *Nitrosospira tenuis* as reference bacterium.

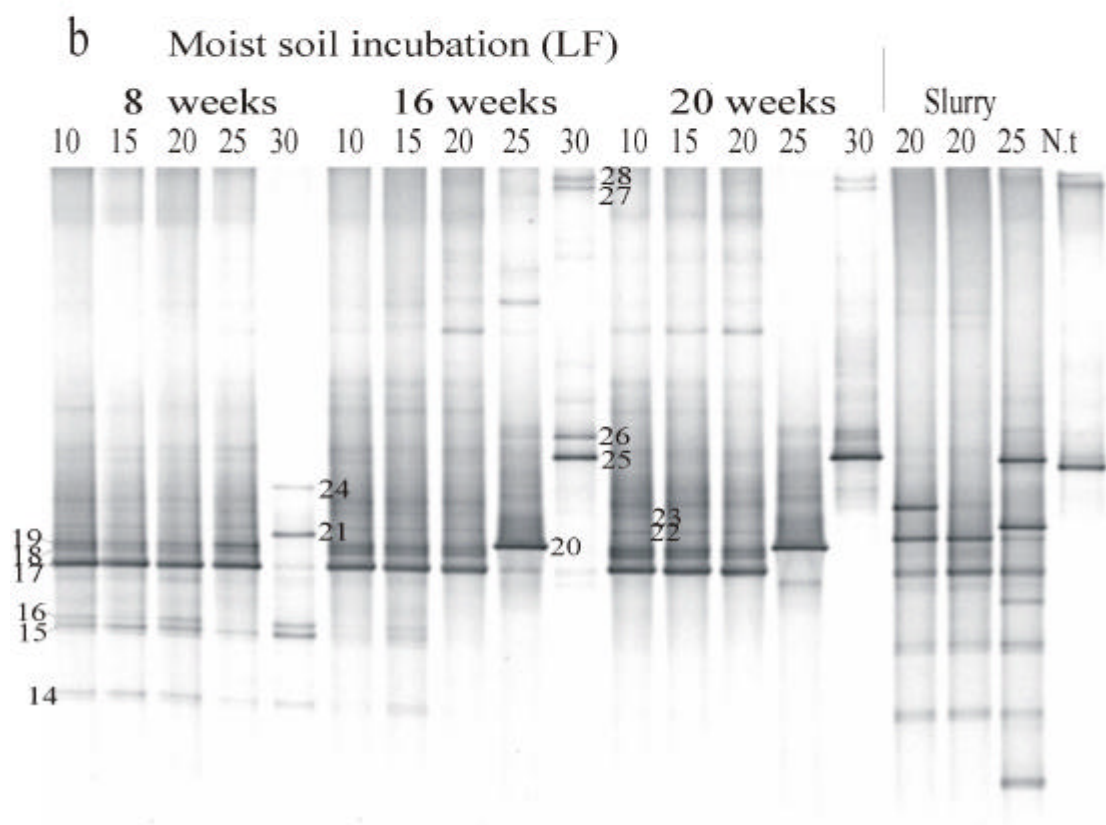
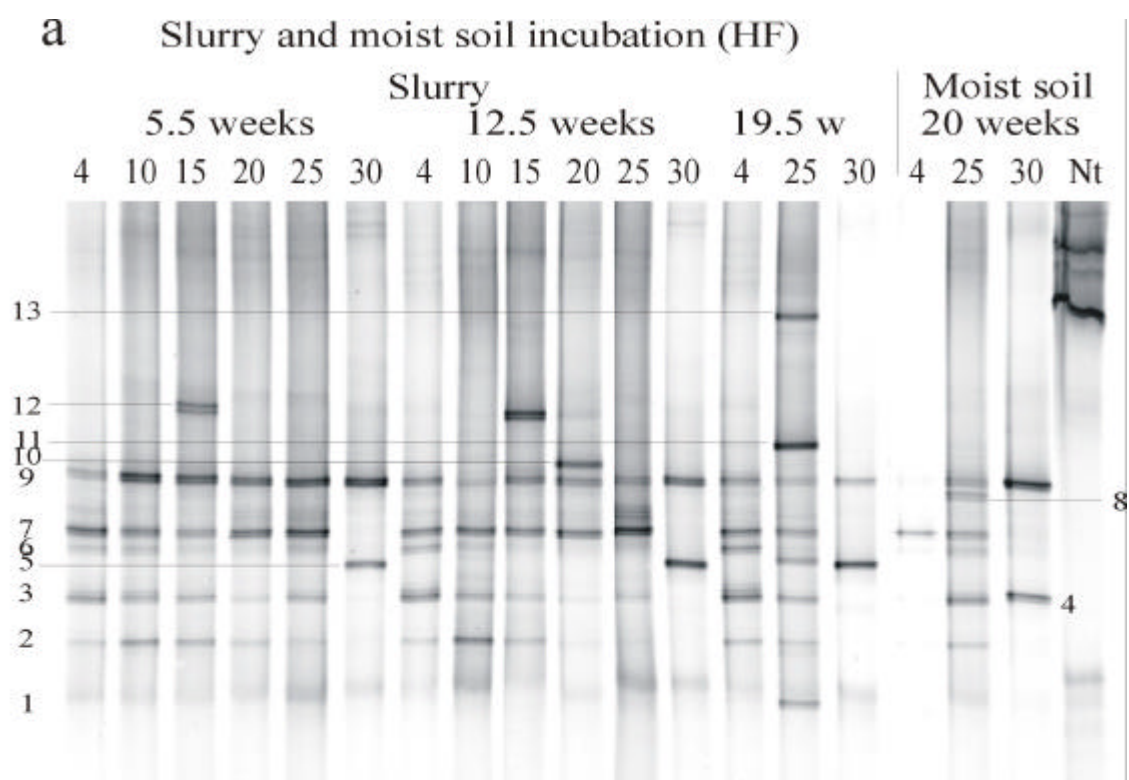


Figure 25: (previous page) DGGE analysis of *amoA* fragments retrieved from OMS soil with (a) high fertilizer (HF) treatment or slurry treatment and (b) low fertilizer (LF) treatment at different temperatures (4 - 30°C) and incubation time. As a comparison three samples of slurry were loaded on the gel with LF samples. The numbering of the bands corresponds to the derived *AmoA* sequences highlighted in Fig. 21. Nt represents *Nitrosospira tenuis* as reference bacterium.

Soil slurry showed a similar diversity and trend as the HF treatment of moist soil incubation. A community shift occurred in the soil slurry at 30°C. One of the bands at 30°C was band 9 (slOMSt25b9 or OMSHFt30b9), which was identical to band 9 in moist soil treatment. The second band at 30°C was band 5 (slOMSt30b5), which was not detected in any other treatments. Sequences of the two bands at 30°C grouped in *Nitrosospira* cluster 3a. In contrast to the incubation at 30°C, DGGE patterns at temperatures between 4 to 25°C showed four major bands, which again were similar between slurry and moist soil in HF treatments (bands 2, 3, 7 and 9; OMSHFt25c16, OMSHFt25b3, OMSHFt25b7 and OMSHFt30b9, respectively) (Fig. 25a). Two additional bands were detected at 20°C or at 15°C only in one of the duplicate incubations (band 10 and band 12: slOMSt25c35, in *Nitrosospira* cluster 1; and slOMSt20b12, in *Nitrosospira* cluster 3b; respectively). After 19.5 weeks of slurry incubation, diversity at 25°C increased and included 3 additional bands (band 1, 11 and 13: slOMSt25b1, in *Nitrosospira* cluster 1; slOMSt25c1, *all*-like sequence; and slOMSt25b13, in *Nitrosospira* cluster 9).

The community structure of OMS soil in the moist LF treatment was different than that in the moist HF or the slurry treatment. However, similar to these treatments a temperature-related community shift occurred in LF moist treatments (Fig. 25b). At 25°C a community shift occurred only after 16 weeks of incubation, and was characterized by a new dominant band (band 20, OMSLFt25b20, identical to *Nitrosospira* AP. AHB1 by amino acid, in *Nitrosospira* cluster 2), which was not detected at any other temperature. However, a community shift at 30°C occurred twice during the incubation period. The first shift occurred after 8 weeks incubation, with sequences of new bands grouping in *Nitrosospira* cluster 1

(bands 14 and 15 OMSLFt15b14 and OMSLFt20b15, respectively), in *Nitrosospira* cluster 3a (band 21: OMSLFt10b21) and in *Nitrosospira* cluster 9 (band 24: OMSLFt30b24). The second community shift occurred after longer incubation (16 weeks). The number of bands decreased and the corresponding sequences grouped in *Nitrosospira* cluster 9 (bands 25 and 26: OMSLFt30b25 and OMSLFt30b26, respectively). Two bands (bands 27 and 28) could not be identified. These bands were suspected to be an artefact, since they could not be re-amplified after excision from the DGGE gel. Cloning of the original sample using either amoAR1 or amoAR3 as reverse primer, and screening of ca. 100 clones by DGGE could not detect clones, which were identical to these two bands. The DGGE banding pattern in samples at 10-20°C did not change during the incubation period of 20 weeks (with the exception of bands 14-15, see below). Most of the sequences at these temperatures were all-like (bands 17, 18, 22, 23: OMSLFt10b17, OMSLFt20b18, OMSLFt10c44 and OMSLFt20c14, respectively). An exception was band 19 (OMSLFt10c2), which was identical by amino acid to *Nitrosospira* sp. B6, in *Nitrosospira* cluster 1.

HF and LF treatment were a selective factor for different communities of ammonia oxidizers. While bands 14-15 (OMSLFt15b14 and OMSLFt20b15, in *Nitrosospira* cluster 1, respectively) were detected in the lower part of the gel at all temperatures of LF treatment after 8 weeks incubation, they were no longer detected after 20 weeks of incubation. Furthermore, although three of the four major bands detected in HF treatments, were identical to sequences detected in LF treatments (bands 3, 7 and 9 identical to bands 15, 17 and 21: OMSLFt20b15, OMSLFt10b17 and OMSLFt10b21, respectively), bands 3 (15) and 9 (21) (in *Nitrosospira* cluster 1 and 3a, respectively) disappeared after longer incubation and only band 7 (17) (all-like sequence) was dominant in slurry, LF and HF treatments.

Correspondence analysis showed that temperature was selecting for different communities. However, the effect of ammonium was much stronger (Fig. 26). Temperature-related community changes (especially in 25°C and 30°C) were observed in LF treatment. In LF treatments, band 20 (in *Nitrosospira* cluster 2) had a large contribution at 25°C, while bands

25-26 (in *Nitrosospira* cluster 9) had a large contribution at 30°C. Separate correspondence analysis for HF treatment and slurry also supported a trend of individual bands with temperature (not shown). However, correspondence analysis did not add additional information to the observation shown by DGGE banding pattern and thus data are not shown.

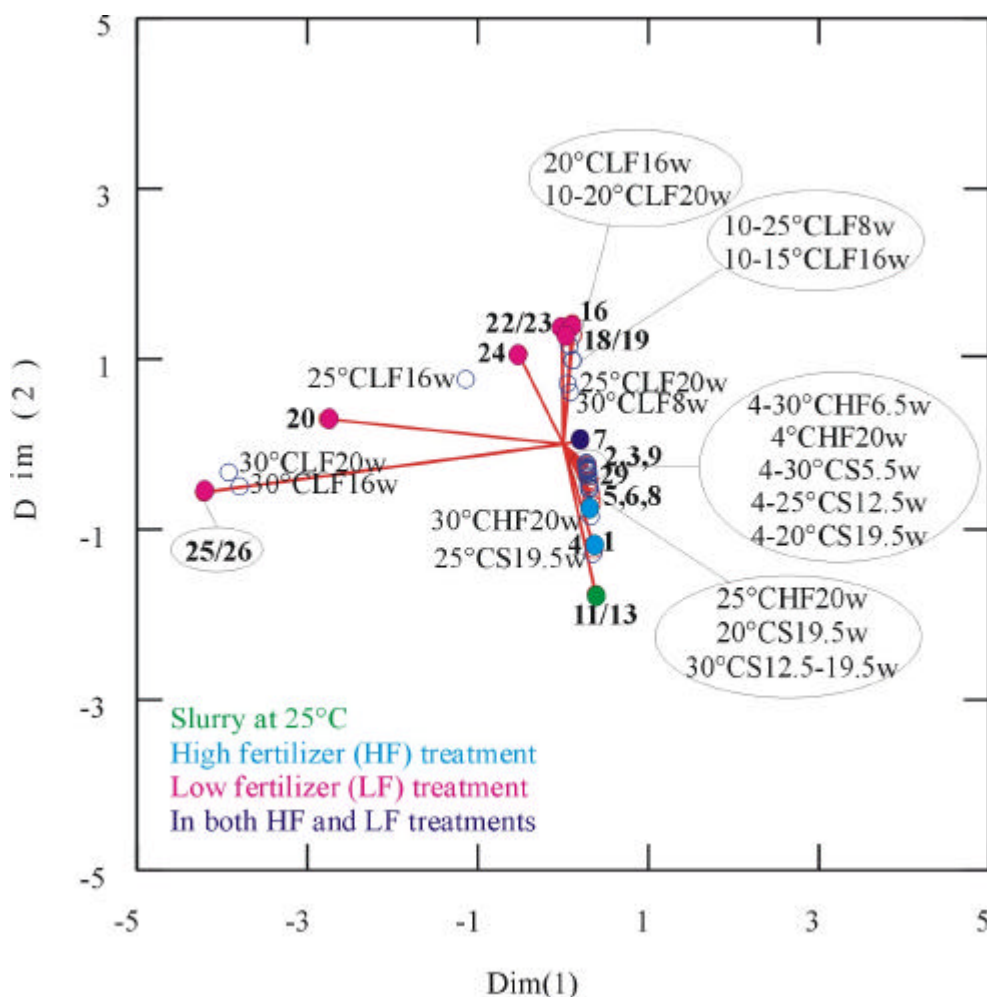


Figure 26: Correspondence analysis comparing the differences in DGGE banding patterns by the program SYSTAT 9. Open circles represent different samples of OMS soil, which were incubated at different temperatures (4-30°C) and/or in different ammonium treatments. Name of samples composed of °C - temperature, S - slurry, LF - low fertilizer treatment, HF - high fertilizer treatment and w - period of incubation in weeks. Filled circles with a line represent DGGE bands with their numbers in bold. Bands 14, 15, 17 and 21 are not shown since they are identical to bands 4, 3, 7 and 9, respectively, but their contribution to the samples is considered in the calculations.

Discussion

The effect of temperature on community structure of ammonia oxidizer was studied using three meadow soils. The diversity of ammonia oxidizers after field sampling of KMS soil (Israel) was low and the sequences grouped mainly in *Nitrosospira* cluster 3a, while the diversity of OMS and GMS soils (Germany) was higher. The majority of sequences from GMS soil grouped in *Nitrosospira* cluster 1 and 4, while sequences from OMS soil grouped in *Nitrosospira* cluster 1, 3b and al1-like sequences. Although OMS soil was acidic (pH 5.0) no bands representing *Nitrosospira* cluster 2 were detected in this soil. *Nitrosospira* AP. AHB1, which has been isolated with acidic medium, as well as environmental sequences and enrichment cultures from acidic soils (pH 4.2) all grouped in this cluster (Stephen et al., 1996; 1998; Kowalchuk et al., 2000b). Also, Stephen et al. (1998) showed a positive correlation with soil acidity and intensity of hybridization signal from DGGE bands representing sequences from *Nitrosospira* cluster 2. However, in agreement with our study, two other native soils with pH 3.35 and 5.4 showed a high diversity represented by 16S rDNA environmental sequences grouping in *Nitrosospira* cluster 1, 3, 4, 6 and 7 (Bruns et al., 1999; Webster et al., 2002), but not in *Nitrosospira* cluster 2. Moreover, recently Carnol et al. (2002) found a dominance of *Nitrosomonas* species in an acidic forest soil. Therefore, the appearance of *Nitrosospira* cluster 2 probably depends on other factors in addition to low pH. However, after incubation of OMS soil at 25°C in LF treatment one band belonging to *Nitrosospira* cluster 2 was observed. Our finding is in agreement with that of Kowalchuk et al. (2000b), who observed development of *Nitrosospira* cluster 2 in MPN at 27°C at acidic conditions and low ammonium concentration (5mM ammonium), although the dominant population in the field sample was *Nitrosospira* cluster 3. The low values of potential nitrification activity observed in OMS soil both in LF and HF treatments are probably due to the acidity of the soil, in agreement with other studies of acidic soils (Tietema, 1992; De Boer and Kester, 1996; Laverman et al., 2000; 2001).

Studying the effect of temperature was done by incubation of the soils in buffered slurry containing urea and in moist soil treated with fertilizer. Although community shifts usually occurred only after a long period of incubation (16 weeks) as observed before in EAS soil (Chapter 2 or Avrahami et al. submitted), a community shift in LF treatment of OMS soil incubated at 30°C occurred already after 8 weeks incubation. Two patterns of community shift due to temperature effect were observed in this study. One pattern was a community shift within *Nitrosospira* cluster 3a, and the second pattern was a community shift between different clusters of *Nitrosospira*. The first pattern of community shift was observed in KMS soil, and was supported by correspondence analysis of the DGGE patterns. Although, different trends of temperature dependency were observed, all representative sequences were grouped within *Nitrosospira* cluster 3a. This was in agreement with the study of EAS soil, where *Nitrosospira* cluster 3 was found at most temperatures, although individual members of this cluster exhibited different trends with temperature (Chapter 2 or Avrahami et al., submitted). The variability within this cluster with respect to temperature is further supported in this study.

The second pattern of community shift due to temperature was observed in GMS and OMS soils, and the shifts were between clusters of *Nitrosospira*. HF treatments shifted from a dominance of *Nitrosospira* cluster 1 and 4, or 1 and all-like sequences at temperatures < 25°C, to a dominance of *Nitrosospira* cluster 3 at 30°C, in HF treatment but not in LF treatment. This observation is in agreement with results of Kowalchuk et al. (2000a; 2000b), who showed that members of *Nitrosospira* cluster 3 were dominant in early successional soils, which have relatively high ammonium concentrations. Furthermore, it is known that cultured strains from *Nitrosospira* cluster 3 grow well in high ammonium culture media (Belser and Schmidt, 1978). However, in EAS soil, *Nitrosospira* cluster 3 was not necessarily dominant at high ammonium concentrations (Chapter 2 or Avrahami et al., submitted). That might imply that individual members of *Nitrosospira* cluster 3 not only show a different trend with temperature but also with respect to ammonium. On the other hand, at 30°C in LF

treatment of OMS soil *Nitrosospira* cluster 9 was the dominant group. Interestingly, *Nitrosospira* cluster 9 was found only at high temperature and low fertilizer concentrations. This is in agreement with EAS soil, where *Nitrosospira* cluster 9 could not be detected at high ammonium concentrations (Chapter 2 or Avrahami et al., submitted), and also with results of Oved et al. (2001), who found this cluster in irrigated agriculture soil, which had been treated with low ammonium concentrations.

Another combination of ammonium and temperature effects was observed in *Nitrosospira* cluster 1, which was one of the dominant groups at temperatures up to 25°C, but was absent at 30°C. In KMS soil, some bands belonging to this cluster appeared at low and at intermediate temperatures (4-20°C). Additional explanation for the appearance of *Nitrosospira* cluster 1 after long incubation in KMS soil might be the observed decrease in pH (from pH 7.9 in the field to values of 6.2-6.6). However, *Nitrosospira* cluster 1 was not observed at temperatures above 20°C in KMS soil, suggesting that this cluster was strongly influenced by temperature. However, since *Nitrosospira* cluster 1 disappeared at all temperatures in LF treatment, it was apparently also influenced by ammonium. The dominance of this cluster in EAS soil at 4-10°C (Chapter 2 or Avrahami et al., submitted), but not at the intermediate temperature range, may be explained by the low ammonium concentrations in the intermediate temperatures. Hence, it seems that *Nitrosospira* cluster 1 is relatively sensitive to high temperatures (as 30°C), but also to low ammonium concentrations.

Another group that showed some trend with temperature, although some members were found at all temperatures, was *Nitrosospira* cluster 4. This cluster was relatively dominant at 25°C. This dominance could not be explained by the finding of Kowalchuk et al. (2000a; 2000b), where *Nitrosospira* cluster 4 was dominant in old successional soils with low ammonium concentration, since ammonium concentrations at 4°C in our study were lower than at 25°C. It might imply that temperature was the actual selective factor and not ammonium. Interestingly, the slurry sample of OMS soil exhibited a higher diversity at 25°C than at the other temperatures, and were characterized by members of *Nitrosospira* cluster 1,

9 and all1-like sequences. This implies that when ammonium is not a limiting factor, although 25°C is not necessarily the optimum temperature for all ammonia oxidizers, it is nevertheless enable the existence of varied species.

The soils sampled in Germany always exhibited a decrease in diversity at 30°C, which was accompanied by a decrease in potential nitrification activity. On the other hand, the increase in activity in HF treatment at 25°C compared to 4°C would not paralleled by an increase of diversity. The main differences between 4°C to 25°C were usually in the intensity of the DGGE bands rather than with the diversity. Surprisingly, KMS soil, which was sampled in Israel, an area with high mean annual temperature, exhibited a rather low diversity at high temperatures (25-37°C). However, the diversity at these temperatures was similar to the diversity in the original soil (*Nitrosospira* cluster 3a). Furthermore, potential nitrification activity at 37°C was not significantly different then at 25-30°C, and higher than at 4°C.

All soils did not show any *Nitrosomonas*-like species independently of the incubation conditions. This was in agreement with previous observations that *Nitrosospira* species are dominant in soils (Kowalchuk et al., 1997; 1998; Stephen et al., 1998; Bruns et al., 1999; Mendum et al., 1999; Phillips et al., 2000; Hastings et al., 2000; Kowalchuk et al., 2000a; 2000b). On the other hand, Phillips et al. (2000), detected *Nitrosomonas*-like sequences in MPN DNA, but not in the DNA extracted directly from the soil. This discrepancy could be explained by using a medium containing urea for our slurry incubation. Aarka et al. (1999b) observed that a medium containing urea was supporting the growth of *Nitrosospira* spp., while a medium containing ammonium sulfate was supporting growth of *Nitrosomonas* spp..

In conclusion, a community shift as a result of temperature effect was observed in the three soils examined as slurry, LF and HF treatments. Two patterns of community shift were observed; within *Nitrosospira* cluster 3a and between different clusters of *Nitrosospira*. *Nitrosospira* cluster 3 showed different trends with respect to temperature and ammonium reflecting the high versatility within this cluster. Some clusters exhibited clear trends with temperature: *Nitrosospira* cluster 4, was dominating at 25°C, *Nitrosospira* cluster 1 was

sensitive to high temperatures (as 30°C), and *Nitrosospira* cluster 9 was found only at high temperatures (25-30°C). Thus, temperature has an effect on the community structure of ammonia oxidizers in soils. The effect of ammonium on community structure of ammonia oxidizers, which had been observed in EAS soil finding (Chapter 3 or Avrahami et al., submitted), was further supported: while members of *Nitrosospira* cluster 1 preferred high ammonium concentrations, those of *Nitrosospira* cluster 9 were mainly found at low ammonium concentrations.

Outlook

Studying some aspects of the ecology of ammonia oxidizers, one of the important groups in the nitrogen cycle, revealed important new information concerning their response to ammonium concentration and temperature. One important factor, which one should consider during investigation the effects of environmental factors on community structure of ammonia oxidizer populations, is the long incubation period (16 to 20 weeks) that is necessary to detect shifts. Moreover, although the presence of ammonium is necessary to support the growth of ammonia oxidizers, ammonium concentration is also an important selective factor for different communities of ammonia oxidizers as was observed in two of our soils (EAS and OMS). The similarity between *amoA* sequences belonging to different ammonia oxidizers is high (> 71% between all ammonia oxidizers within β -*Proteobacteria* i.e. *Nitrosospira* and *Nitrosomonas*) and even higher within *Nitrosospira*, the dominant population in soils (> 89% among pure culture). Nevertheless, there are marked differences between *Nitrosospira* clusters and even within clusters (like *Nitrosospira* cluster 3) in their response to environmental factors like ammonium concentration and temperature. The similarities between different species within *Nitrosospira* cluster 3 are lower than between species within other clusters of *Nitrosospira*. Therefore, it is not surprising that this cluster shows higher variability both with respect to ammonium concentrations and temperature. This study showed the importance of using a combination of two factors (temperature and ammonium) and a similar approach should be considered while new experiments are planned. Furthermore, studying several environments with different characteristics, also provides a more comprehensive picture, and therefore is recommended in the future. In addition to moderate environments, we can learn more about the selection pressure in extreme environments, such as soils from the north latitude or from warm locations like Sahara desert. Further studies, which would address the effect of other environmental factors on the community structure of ammonia oxidizers, would elucidate our understanding the distributions of ammonia oxidizers in different environmental conditions.

The molecular tools to study community shifts of ammonia oxidizer in soils were improved in this work by the non-degenerate reverse primer amoAR1. However, further improvement of the present primer set (amoA-1F/amoAR1) is still necessary to enable the detection of *Nitrosospira* cluster 10. This cluster was detected by amoA-2F/amoA-5R, but not with our primer system (amoA-1F/amoAR1) while it was in a mixture of clones. Using the molecular tools to study the diversity of ammonia oxidizers contributes to our understanding of their distribution in the environment. However, the molecular tools alone are not enough. Thus, in spite of the difficulties, isolation of ammonia oxidizers identified by molecular methods is still very important to obtain a more comprehensive picture of their phenotypes. Using both enrichment and isolation together with the molecular techniques in extreme environment might result in new species, or known ammonia oxidizers, which are able to survive in such conditions. Furthermore, there are still clusters where no isolates are available. One example for such a group is *Nitrosospira* cluster 9. This study showed clearly that sequences retrieved from this cluster prefer high temperature and low ammonium concentrations. Using this knowledge during the attempts to cultivate species belonging to this cluster might help in their isolation. Another important fact that might help in the isolation of this group is that other groups of ammonia oxidizers like *Nitrosospira* 1 do not survive at high temperatures. So, the combination of these facts would be important in the decision which environmental sample would be preferable for the first inoculation.

Ammonia oxidizers are also important in other environments and from previous studies it is known that similar to this work different clusters of *Nitrosomonas* showed opposite trends with respect to ammonium. However, temperature effect was not studied yet in lakes, marine environments, and activated sludge, which are dominated by *Nitrosomonas*-like species as well as member of *Nitrosospira* cluster 1 defined by 16S rRNA. We cannot rule out the possibility that *Nitrosomonas* species would also reveal differences with their responds to temperature and thus studying the effect of temperature is a promising task also in other environments.

Summary

Temperature was found to play an important role in oxidation of ammonium in soils by influencing indirectly (i.e. by ammonium concentration) the activity rates. Temperature was also influencing directly and indirectly the community structure of ammonia oxidizers bacteria. The effect of temperature on N_2O release was studied, in order to find a conceivable explanation for the low NO and N_2O production rates after short-term adaptation to intermediate temperatures (Gödde and Conrad, 1999). Using the same agriculture soil (EAS) showed that as long as ammonium was not limiting, release rates of N_2O monotonously increased between 4 and 37°C after short-term temperature adaptation, with nitrification accounting for about 35-50% of the N_2O production between 4 and 25°C. A longer period of incubation (6.5, 16 and 20 weeks) at different temperatures (4-37°C) resulted in a decrease of ammonium concentrations that was more pronounced at temperatures between 10-25°C than at either 4°C or 37°C. Consistently, potential nitrification activity was higher between 10-25°C than at either 4°C or 37°C. Collectively these results indicate that ammonium was a limiting factor at the intermediate temperatures during the experiments by Gödde and Conrad, (1999) but not in this study. Therefore, a shift in the community of ammonia oxidizers is not required to explain the minimum of N_2O release at the intermediate temperatures found by Gödde and Conrad (1999). Indeed, analysis of the community structure of ammonia oxidizers after short incubation at different ammonium concentrations did not show any community shift after 4 weeks of incubation, although nitrification activity increased upon addition of ammonium.

In order to demonstrate potential effects of temperature and fertilization on the composition of the soil ammonia oxidizer community, the same EAS soil was studied in moist state during long incubation (20 weeks) at low (0.3%) and high (1%) concentration of commercial fertilizer. Since temperature seems to influence the soil ammonium concentrations after a short incubation, it was assumed that any effect of temperature would have to be tested at different ammonium concentrations. The soil was therefore also incubated

in buffered (pH 7) slurry amended with urea in which ammonium concentrations and pH were better controlled. Communities of ammonia oxidizers were assayed by denaturant gradient gel electrophoresis (DGGE) of the *amoA* gene coding for the small subunit of ammonia monooxygenase. Optimization of the *amoA* PCR system by testing non-degenerate primers was necessary, since similar to Nicolaisen and Ramsing (2002) multiple DGGE bands were observed due to the two wobble positions in the *amoA* reverse primer, i.e. *amoA*-2R (Rotthauwe et al., 1997). A PCR system using a non-degenerate reverse primer (*amoAR1*) gave the best results with our samples. The primary conclusion drawn from these incubations was that the community shifts were clearly influenced by the different fertilizer treatments, indicating that ammonium was a selective factor for different ammonia oxidizer populations. Although in correspondence analysis the effect of fertilizer treatment masked that of temperature, correspondence analysis separately for each treatment revealed the effect of temperature on community structure. Moreover, community shifts were also observed in the soil slurries, in which ammonium concentrations and pH had been kept within a relatively narrow range.

The effect of temperature on community structure of ammonia-oxidizing bacteria was further investigated in three meadow soils, which differed in pH, mean annual temperature and original diversity of ammonia oxidizers. The assumption was that sampling from locations with different mean annual temperature would increase the probability to detect temperature-adapted populations. Two of the soils (OMS and GMS) were acidic (pH 5.0 - 5.8) and from sites with low mean annual temperatures (9.9 ± 1.9 °C, Germany), while KMS soil was slightly alkaline (pH 7.9) and from a site with higher mean annual temperature (22.2 ± 1.8 °C, Israel). The soils were incubated at different temperatures (4 - 37 °C) in moist state and in slurry for 20 weeks and the community structure was analysed as before. Two patterns of community shifts were observed: One pattern was a community shift within *Nitrosospira* cluster 3. This cluster was found at all temperatures, but individual bands exhibited a trend with temperature. This pattern was found in the Israeli soil. The second pattern showed a

community shift between *Nitrosospira* clusters. *Nitrosospira* cluster 1 was mainly detected below 30°C, while *Nitrosospira* cluster 4 was predominant at 25°C. *Nitrosospira* cluster 3 dominated at 30°C in high fertilizer (HF) treatment, while *Nitrosospira* cluster 9 dominated at 30°C in low fertilizer (LF) treatment. This pattern was observed in the German soils. It was concluded that ammonia oxidizer populations are influenced by temperature. Furthermore, low fertilizer treatment of OMS soil confirmed that N-fertilizer also influenced the diversity of ammonia oxidizers. Thus, *Nitrosospira* cluster 1 was absent at low ammonium concentrations, while *Nitrosospira* cluster 9 was only found at these concentrations.

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Curriculum Vitae

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Education:

1982-87 – “Denmark” high school, Jerusalem, Israel: specialization in Biology.

1987-88 – Volunteer service in the ‘ The Hebrew Scouts’.

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1992-94 – B.Sc. in Life Sciences at Tel Aviv University. Final year research project: “Characterization of bacteria that induce and inhibit growth in lettuce seeds.” Supervisor: Prof. Jacob Freedman

1995-97 – M.Sc. in Natural Sciences at the Ecology Systematic and Evolution Department, the Hebrew University of Jerusalem. Thesis: “Phosphatase and phosphodiesterase in halophilic bacteria and their importance in adaptation to life in the Dead Sea.” Supervisor: Prof. Aharon Oren

September 1997–November 1997 - “Max Plank Institute for Marine Microbiology”, Bremen, Germany. Project: Studying the diversity of microorganism in Solar Lake, Sinai, with molecular tools (DGGE, cloning and RNA hybridization). Supervisors: Dr. Dror Minz and Dr. Gerard Muyzer.

1997–99 – The Hebrew University of Jerusalem. Research on: “Ammonia-oxidizing bacteria spatial and temporal distribution and diversity in monomictic and hypersaline lake: Solar Lake.” Supervisor: Prof. Yehuda Cohen.

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2000 – Present – Doctoral research in Microbial Ecology at “Max Planck Institute for Terrestrial Microbiology”, Marburg, Germany, under supervision of Prof. Ralf Conrad. Thesis: “Effect of temperature and soil ammonium concentration on activity and diversity of ammonia oxidizers bacteria”. Planned submission and defence of my thesis is December, 2002.

Publications and Scientific Presentations:

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A paper for the book of the above conference:

S. Avrahami and A. Oren. 1996. “Phosphatase and phosphodiesterase activities in salt-saturated environments: The Dead Sea and Saltern Crystallizer ponds”. In: Preservation of Our World in the Wake of Change volume 6 B. ISEEQS Publication. Jerusalem, Israel. Page: 810-813.

“Effect of temperature on activity and community structure of ammonia-oxidizers in two meadow soils”. A short talk presented at the FAAM meeting, Goettingen, Germany, March 2000.

Avrahami, S., G. Braker and R. Conrad. 2002. Effect of soil ammonium concentration and temperature on N₂O release and the community structure of ammonia oxidizers and denitrifiers. Applied Environmental Microbiology. 68: 5685-5692

Avrahami, S., W. Liesack, and R. Conrad. Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. Submitted.

Avrahami, S. and R. Conrad. Long-term effect of temperature on community structure of ammonia oxidizers in different meadow soils. In preparation.

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Pledge

I certify that the present thesis entitle:

‘Effects of temperature, soil ammonium concentration and fertilizer on activity and community structure of ammonia oxidizers’

was carried out without any unlawful device. I did not use any other than the described literature sources or technical devices.

This work has never been submitted before in this or similar form to any other university and has not been used before any examination.

Marburg 18.12.2002

Sharon Avrahami